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Bedeutung der Cycline für die kolorektale Karzinogenese

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Zielsetzung: In der vorliegenden Arbeit sollen Cycline, den Zellzyklus und damit die Proliferation und/oder die Differenzierung steuernde Gene, sowie verwandte Gene in unterschiedlichen Stufen der intestinalen Karzinogenese untersucht werden.

Patienten und Methodik: Primäre Kolonadenome, Kolonkarzinome aller Stadien und korrespondierende normale Kolonmukosa sowie Dünndarmkarzinome wurden anläßlich chirurgischer und endoskopischer Eingriffe von Patienten gewonnen, die in eine Gewebeentnahme einwilligten. Ergänzend hierzu wurden etablierte Zellinien kolorektaler Karzinome sowie eine intestinale Epithelzelline und durch retrovirale Transduktion K-ras- oder Cyclin D1-überexprimierende Klone dieser Zellinie untersucht. Die Analysen erfolgten mittels molekularbiologischer Methoden, insbesondere anhand von Western- und Northern-Blot-Analysen, sowie unter Verwendung der Polymerase-Kettenreaktion. Zur Sicherung der erhaltenen Ergebnisse wurden immunhistochemische Untersuchungen des Ausgangsgewebes durchgeführt.

Ergebnisse: 1) Mutationen im Codon 12 des K-ras-Protoonkogens sind in Adenokarzinomen des Dünndarms in ähnlicher Häufigkeit nachzuweisen wie in Kolontumoren. 2) Eine Aktivierung des K-ras-Protoonkogens bewirkt eine Cyclin D1-Überexpression in intestinalen Epithelzellen 3) Die G1-Phase-Cycline D1 und E weisen in Kolonkarzinomen überwiegend sowohl eine Erhöhung ihrer mRNA- als auch ihrer Proteinexpression auf. 4) Die konstitutive Überexpression von Cyclin D1 in intestinalen Epithelzellen hat weder einen Einfluß auf das Wachstumsverhalten dieser Zellen in vitro noch führt es zur Tumorgenität der Cyclin D1 überexprimierenden Klone in vivo. 5) Eine Überexpression von Cyclin E-Proteinen liegt in Kolonkarzinomen, nicht jedoch in deren Vorstufen vor und ist damit eng mit der Transformation kolorektaler Epithelzellen assoziiert. 6) Die Cyclin E-Protein- und mRNA-Expressionen kolorektaler Karzinome sind nicht miteinander korreliert. 7) In kolorektalen Karzinomen besteht ein Zusammenhang zwischen einer, im Vergleich zu normaler Kolonmukosa, erhöhten bzw. aberranten Cyclin E-Proteinexpression und dem Mutator-Phänotyp dieser Karzinome.

Schlußfolgerung: In kolorektalen Tumoren ist die Cyclin D1-Überexpression teilweise Ausdruck des bereits in der Frühphase der Karzinogenese aktivierten K-ras-Protoonkogens. Cyclin D1 wirkt selbst nicht als Onkogen. Im Gegensatz hierzu scheint eine Überexpression von Cyclin E die maligne Transformation kolorektaler Tumoren zu unterstützen. Ferner könnte eine Erhöhung der Cyclin E-Proteinexpression die DNA-Replikation stören und damit bereits defekte DNA-Reparatur-Mechanismen verstärken.

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1 Einleitung

1.1 Kolorektales Karzinom

1.1.1 Anatomie, Pathologie

Die Kolonmukosa ist aus einzelnen Krypten aufgebaut, welche überwiegend von Epithelzellen ausgekleidet werden. Die ständige Regeneration der Krypten steht in einem Gleichgewicht zwischen Proliferation der an der Basis lokalisierten Stammzellen und einer stufenweise Differenzierung der von der Basis zur Kryptenspitze wandernden Epithelzellen. Untersuchungen von Lipkin zeigten, dass dieser Vorgang ca. 20 Stunden dauert. Dünndarmepithelzellen benötigen hingegen 3 Tage bis sie das Darmlumen erreichen und abgeschilfert werden (34, 52).

Ist dieser Prozeß zugunsten einer gesteigerten Proliferation und/oder mangelnden Differenzierung gestört, kommt es zur Hyperplasie und möglichen malignen Entartung der Schleimhaut. Erste Stufen der Hyperplasie sind mikroskopisch erkennbare "aberrante Krypten" (ACF, aberrant crypt foci), die sich im Zeitraum von mehreren Jahren zu makroskopisch erkennbaren Adenomen unterschiedlicher Dysplasiegrade entwickeln und in ein Karzinom (Abb. 1) übergehen können (83)

Neben dieser Adenom-Karzinom-Sequenz, die für den überwiegenden Anteil der kolorektalen Karzinome verantwortlich ist, besteht die Möglichkeit der "de novo"-Entstehung kolorektaler Karzinome aus sogenannten "flat adenoma". Dies spielt vor allem bei der Kolonkarzinogenese auf dem Boden chronisch-entzündlicher Darmerkrankungen und bei den hereditären nicht-polypösen kolorektalen Karzinomen (HNPCC–Syndrom) eine Rolle (56, 107).

Kolonkarzinome werden nach ihrer intramuralen Ausdehnung und ihrer Metastasierung in lokoregionäre Lymphknoten sowie in Organe wie z. B. Leber und Lunge nach Dukes (25) bzw. der UICC–Klassifikation in prognostisch relevante Stadien eingeteilt (115). Voraussetzung für die Beurteilung des Lymphknotenstatus ist, daß mindestens 12 lokoregionäre Lymphknoten auf eine Tumorinvasion hin

untersucht werden. Der Differenzierungsgrad des Karzinoms wird nach Grad 1 (gut differenziert) bis Grad 4 (undifferenziert) beurteilt. Histologische Sonderformen des kolorektalen Karzinoms stellen verschleimende Karzinome sowie das seltene Siegelring-Karzinom und Karzinome medullärer Differenzierung dar (80).

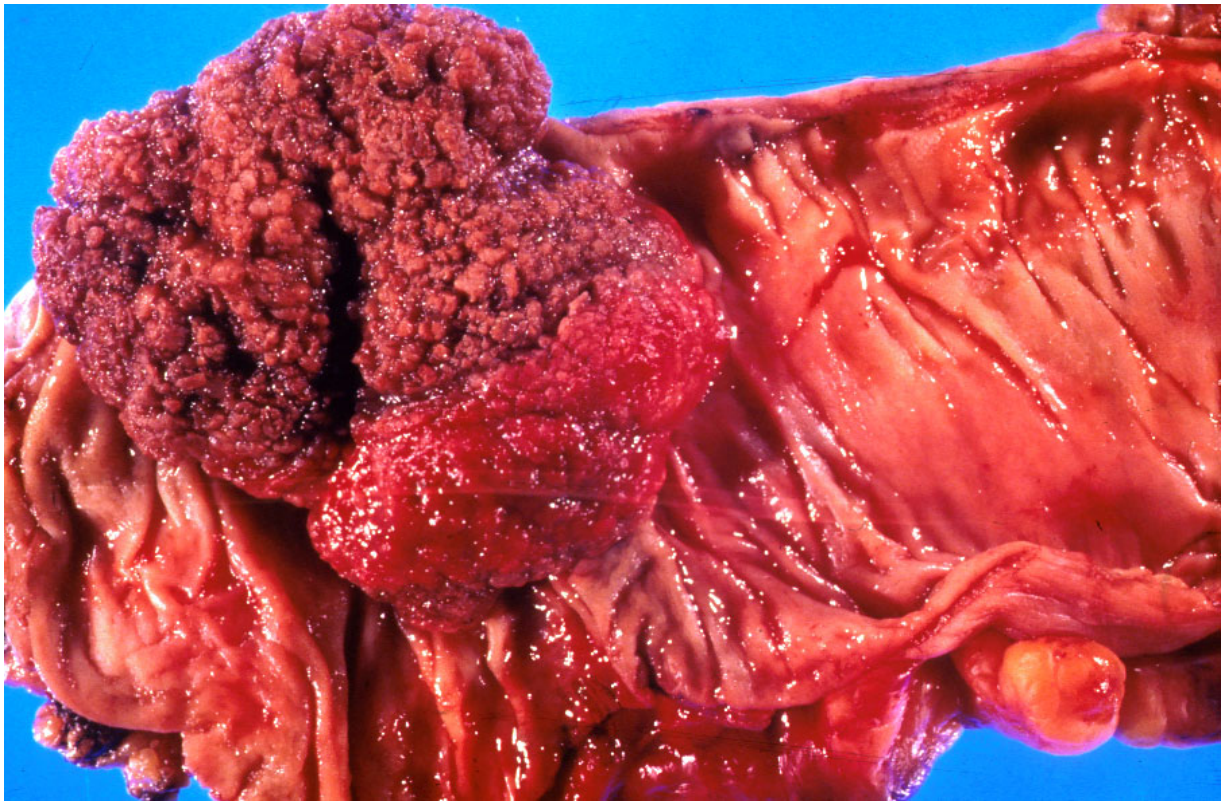

Abb. 1: Polypöses Kolonkarzinom pT3, pN1, M0, G2 (Dukes C, UICC Stadium III)

1.1.2 Epidemiologie

Die Inzidenz des kolorektalen Karzinoms hat sich in Deutschland von 1960 bis 1980 verdoppelt und beläuft sich derzeit auf ca. 50.000 Neuerkrankungen/Jahr. Kolorektale Karzinome stellen mit jährlich ca. 30.000 Todesfällen die zweithäufigste tumorbedingte Todesursache in Deutschland dar. Diese Rangfolge gilt allgemein für westliche Industrieländer und ist auf Risikofaktoren wie eine fettreiche und ballaststoffarme Ernährung, Alkoholkonsum und eine mangelnde körperliche Aktivität

zurückzuführen. Protektiv gegenüber der Entstehung kolorektaler Karzinome wirken u. a. Ballaststoffe, Calcium und Acetylsalicylsäure. Der Erkrankungsgipfel liegt im 7. Lebensjahrzehnt (83).

Die Ausbildung von Kolonkarzinomen ist das Ergebnis einer Wechselwirkung von Umweltfaktoren und genetischer Prädisposition. Neben den genannten Ernährungsfaktoren liegt bei bis zu 50 % der Kolonkarzinome eine genetische Prädisposition unterschiedlicher Ausprägung vor. In ca. 5 % der kolorektalen Karzinome ist ein autosomal–dominanter Erbgang zu beobachten. Dabei handelt es sich in 1 % der kolorektalen Karzinome um eine Keimbahnmutation im APC-Gen, welche für die Manifestation der Polyposis coli (Abb. 2) verantwortlich ist. Eine Rarität stellt das Peutz–Jeghers–Syndrom mit autosomal–dominanter Vererbung einer Mutation im Peutz–Jeghers-Gen, einer Serin/Threoninkinase, dar. Der größte Anteil familiärer kolorektaler Karzinome entfällt auf das HNPCC–Syndrom, welches ca. 4 % der kolorektalen Karzinome ausmacht (1, 31, 56, 106). Ursache des HNPCC–Syndroms sind Keimbahnmutationen in DNA–Reparatur–Genen, namentlich im hMLH1- (17) oder hMSH2–Gen (30). Mutationsträger haben ein Risiko von 80%, an einem kolorektalen Karzinom zu erkranken. Hinzu tritt ein weiteres, wenn auch geringeres Risiko für HNPCC–assoziierte extrakolonische Karzinome wie z. B. Magenkarzinome, Endometriumkarzinome und Karzinome der ableitenden Harnwege. Epidemiologische Untersuchungen in HNPCC-Familien unterschiedlicher Kulturkreise zeigten verschiedene Spektren extrakolonischer Tumoren (69). Diese Beobachtung verdeutlicht die wechselseitige Beziehung von genetischer Disposition und Umwelt- bzw. Ernährungsfaktoren.

Sonderformen kolorektaler Karzinome stellen Karzinome dar, die auf dem Boden einer chronisch-entzündlichen Darmerkrankung, insbesondere der Colitis ulcerosa entstehen. Hier steigt das Risiko eines Karzinoms linear mit der Erkrankungsdauer an und beträgt nach 30 Jahren 18 % (26).

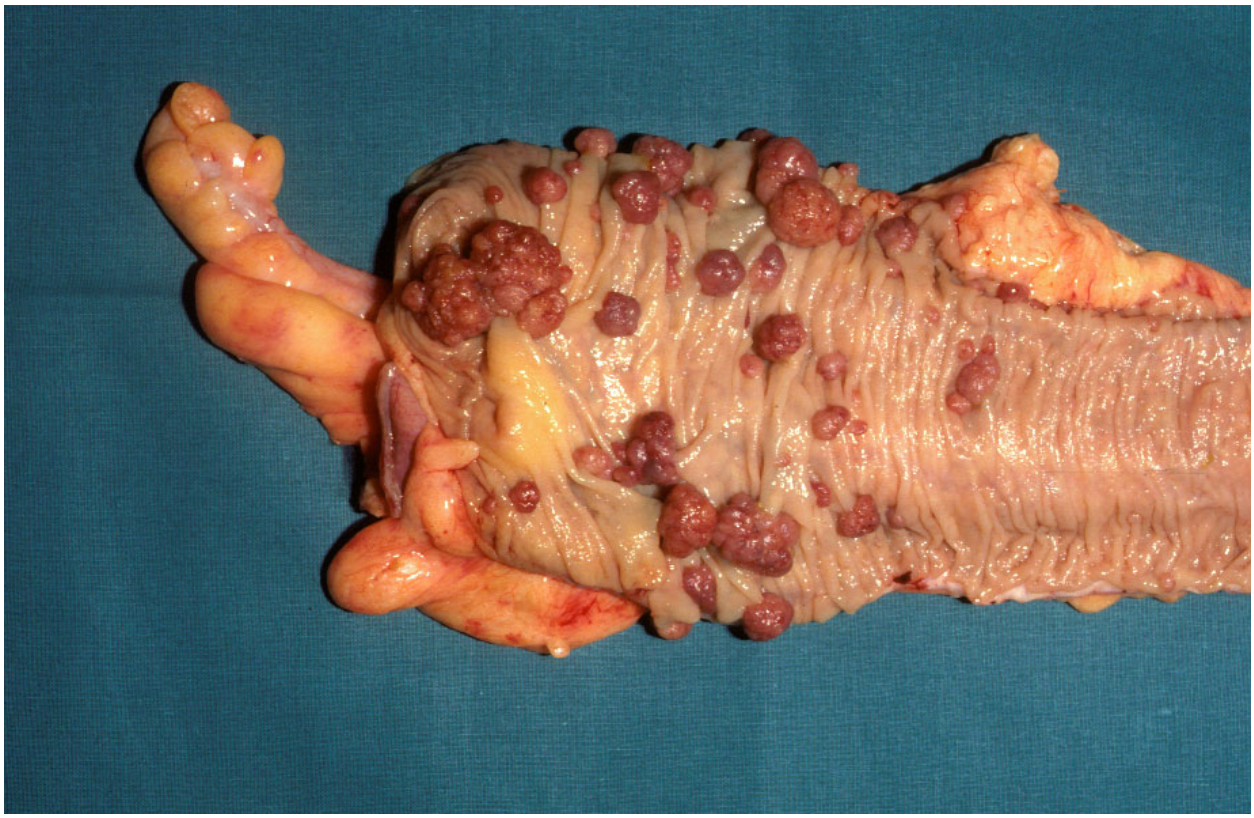

Abb. 2: Attenuierte Form einer familiären Polyposis coli, APC-Mutation Exon 9 Codon 332 (Arg 332 ter)

1.1.3 Prävention

Eine wichtige Säule in der Prävention des kolorektalen Karzinoms ist der endoskopische Nachweis und die Entfernung von Kolonadenomen. Da bis zu 2/3 der Kolontumoren innerhalb einer Woche bluten, wird die 3-malige wiederholte Testung von Stuhlproben auf Blut (Hämoccult ®-Test) ab dem 45. Lebensjahr empfohlen. Ein positiver Hämoccult-Test stellt die Indikation zur kompletten Koloskopie dar. Eine regelmäßige Durchführung des Tests kann die Mortalität bezüglich des kolorektalen Karzinoms um ca. 20% senken. Diskutiert werden zur Zeit eine erweiterte Vorsorge mittels Sigmoidoskopie (ab dem 50. Lebensjahr) in 5-Jahres-Intervallen und die routinemäßige Koloskopie (ab dem 55. Lebensjahr) in 10-Jahres-Abständen, entsprechend dem Intervall der Adenom-Karzinomsequenz sporadischer Kolonkarzinome (19).

Im Vergleich zum Hämoccult®-Test besitzen endoskopische Verfahren eine wesentlich höhere Sensitivität und vor allem eine höhere Spezifität. Die Sigmoidoskopie hat sich als effiziente Screeningmethode erwiesen und konnte in Studien die Mortalität rekto-sigmoidaler Karzinome um 60 – 80% senken. Vorteil der Sigmoidoskopie ist gegenüber der totalen Koloskopie die relativ einfache Durchführung und damit verbundene höhere Patientenakzeptanz. Der Nachteil der Sigmoidoskopie besteht in der fehlenden Beurteilung der proximalen Kolonabschnitte Es wird daher eine Kombination aus Sigmoidoskopie und regelmäßigen Hämoccult®-Tests bzw. einer Koloskopie im Intervall von 10 Jahren empfohlen (64, 83).

Abweichend von dieser Regel müssen sich Patienten mit autosomal-dominanter Vererbung eines kolorektalen Karzinoms (FAP, HNPCC) bereits in jüngerem Alter und in jährlichen Abständen einer kompletten Koloskopie unterziehen. Der Grund hierfür liegt zum einen in dem vorgezogenen Altersgipfel für hereditäre kolorektale Karzinome, zum anderen in der Häufigkeit der Kolonadenome bei FAP bzw. der beschleunigten Karzinomsequenz beim HNPCC-Syndrom. Ähnlich wie beim HNPCC-Syndrom weisen auch Colitis ulcerosa-assoziierte Kolonkarzinome eine beschleunigte Karzinogenese auf, so daß bei Colitis ulcerosa-Patienten ein engmaschiges endoskopisches Monitoring erforderlich ist (26, 44).

Neben der Detektion von präkanzerösen Läsionen haben epidemiologische und molekulare Untersuchungen zu neuartigen Ansätzen der Chemoprävention geführt. Geeignete Substanzen, die lokal oder systemisch inhibitorisch auf die Proliferation und mögliche Transformation normaler Kolonmukosazellen wirken, sind u. a. Acetylsalicylsäure (6), Na-Butyrat (13) und Calcium (86).

Hinsichtlich der Möglichkeiten einer Chemoprävention ist es erforderlich, insbesondere die initialen genetischen und/oder epigentischen Ereignisse der mehrstufigen Kolonkarzinogenese zu identifizieren und Strategien zu entwickeln, diesen entgegenzusteuern. In diesem Zusammenhang ist es von besonderem Interesse, daß bei Erkrankungen mit einem erhöhten Risiko für ein kolorektales Karzinom wie Colitis ulcerosa und HNPCC-Syndrom bereits in mikroskopisch unauffälliger Kolonmukosa chromosomale Aberrationen (76) respektive DNA-

Reparatur-Defekte (22) nachweisbar sind. Diese Phänomene könnten als "intermediate Marker" möglicher Chemoprävention-Programme dienen.

1.1.4 Therapie

Standardtherapie des kolorektalen Karzinoms ist die Resektion des befallenen Darmabschnittes unter präliminärer Ligatur der versorgenden Blutgefäße und unter Mitnahme der regionären Lymphknoten. Eingeschränkt radikale Operationsverfahren dienen der Palliation bei Vorliegen einer Fernmetastasierung oder sind z. B. bei definierten Frühstadien ("low risk-Karzinome") eines Rektumkarzinoms bzw. bei endoskopisch unvollständig entfernten Adenomen indiziert. Lokale Tumorresektionen können mit ausreichender Sicherheit laparoskopisch oder im Falle von Rektumkarzinomen auch durch spezielle mikrochirurgische Verfahren durchgeführt werden (82, 90). Nach der bisherigen Beobachtungszeit wird damit die Sicherheit konventioneller Radikaloperationen kolorektaler Karzinome erreicht (18). Ergänzend zur operativen Therapie wird bei Kolonkarzinomen mit lymphonodaler Metastasierung eine adjuvante Chemotherapie durchgeführt. Inwieweit es sinnvoll ist, ausgewählte Patienten bereits in früheren Stadien einer adjuvanten Chemotherapie zuzuführen, ist derzeit Gegenstand kontrollierter Studien. Wünschenswert wären weitere, z. B. molekulare Prognosemarker, die der Therapiestratifizierung dienten (113).

1.2 Molekulare Karzinogenese

1.2.1 Aktivierung von Protoonkogenen

1914 schlußfolgerte Boveri in Würzburg aus Beobachtungen an Seeigeleiern, daß Änderungen im Chromosomenbestand der Zelle Ursache der Krebsentstehung seien (16). Diese These erweiterte 1928 der Göttinger Chirurg Bauer, aufbauend auf die Mendelsche Vererbungslehre und die Beobachtung durch Röntgenstrahlen

induzierter Karzinome, dahingehend, daß nicht nur chromosomale Aberrationen, sondern bereits Mutationen einzelner Gene für die Karzinomentwicklung verantwortlich seien: „*und wie die normalen Gene als enzymartig wirkende Stoffe die letzten Antriebskräfte aller Zellfunktionen darstellen, so sind mutierte Gene somatischer Zellen die letzten Träger der Geschwulsteigenschaften*“(9). Detaillierter noch, insbesondere hinsichtlich der Bedeutung der Zellproliferation für die Tumorgenese, wies 1934 Lockhart-Mummary, ein bedeutender Vertreter der kolorektalen Chirurgie, auf den Zusammenhang zwischen proliferationsfördernden Genen und der Tumorentstehung hin*: „It would seem probable that the growth rate of somatic cells must be controlled by certain genes contained in the nucleus of the cell, and if these genes have been mutated in such a way that the normal rate of growth is increased, that is to say, if mitosis of the cell occurs at more frequent intervals than is normal for that particular tissue, then it follows that a tumor must result“* (53).

Zur Überprüfung dieser Arbeitshypothesen bedurfte es jedoch der theoretischen und methodischen Voraussetzungen der modernen Molekuarbiologie, welche 1953 durch die Aufklärung der DNA-Struktur durch Watson und Crick begründet wurde (108). Ein entscheidender Durchbruch in der molekularen Tumorforschung gelang 1976 Stehelin, indem er eine Gensequenz des ***s***imian ***r***at sar***c***oma Viruses mit zelltransformierenden Eigenschaften identifizierte, welche das erste dominante Onkogen, namentlich das src-Onkogen, darstellte (95).

Mit Hilfe von Transfektionsexperimenten gelang die Identifizierung einer Reihe ähnlicher, dominanter Onkogene, deren Wildtyp-Sequenz, das Protoonkogen, für Proteine kodierte, die Schlüsselfunktionen in der Übertragung von Proliferations- und/oder Differenzierungssignalen haben und je nach ihrer subzellulären Lokalisation Wachstumsfaktoren, Membranrezeptoren, Kinasen oder Transkriptionsfaktoren zugeordent werden können (10). Ein typischer Vertreter eines solchen dominanten Onkogens ist z. B. das beim kolorektalen Karzinom häufig mutierte K-ras (***K***irstin ***ra***t ***s***arcoma) Gen, welches für ein membranständiges Protein kodiert, welches GTP bindet und hydrolisiert. Eine Punktmutation im K-ras Proto-Onkogen führt zur konstitutiven Aktivierung des Ras-Signalweges durch Reduktion der Ras-abhängigen GTPase-Aktivität (15).

In vitro können nicht-maligne Zellen durch Mutationen von Protoonkogenen oder durch eine ektope, konstitutive Überexpression von Protoonkogenen zu Zellen mit einem malignen Phänotyp transformiert werden. Im transgenen Mausmodell entstehen durch das Einbringen eines dominanten Onkogens in die Keimbahn und die konstitutive Aktivierung proliferationsfördernder Signalwege eine Hyperplasie bzw. Malignome in Organen, in denen der jeweilige Signalweg aktiviert ist (14, 21, 105).

1.2.2 Inaktivierung von Tumorsuppressorgenen

Komplementär zu den dominanten Onkogenen, welche durch die Mutation nur eines von zwei Allelen bzw. durch die Transfektion eines mutierten DNA-Stranges normalen Zellen einen malignen Phänotyp verleihen, d. h. diese Zellen transformieren, stehen die Tumor-Suppressorgene (109). Diese tragen durch Verlust ihrer Funktion zur Krebsentstehung bei. Aufgrund der Tatsache, daß für einen Funktionsverlust beide Allele diploider Zellen durch Mutation bzw. chromosomale Aberration inaktiviert werden müssen, sind dies geeignete Zielgene hereditärer Tumorsyndrome. Entspechend der von Knudson für das Retinoblastom aufgestellten Zweitreffertheorie ist für die Manifestation des Tumors neben der Keimbahnmutation eines Allels, eine somatische Mutation des zweiten Wildtyp-Allels erforderlich (49). Das Retinoblastoma-Gen konnte als erstes Tumorsuppressorgen identifiziert werden. Erst die detaillierte Aufklärung der Zellzyklusregulation zeigte, daß dieses Suppressorgen eine zentrale Rolle in der Steuerung der DNA-Replikation aller menschlicher Tumoren einnimmt. Ein zweites klassisches Suppressorgen ist das ebenfalls eng mit dem Zellzyklus assoziierte und beim Menschen am häufigsten mutierte p53-Gen. Keimbahnmutationen dieses Gens führen zu dem seltenen Li-Fraumeni-Syndrom, einer hereditären Prädisposition für Karzinome mehrerer Organe. Im Gegensatz zu dominanten Onkogenen ist die Funktionsanalyse der Suppressorgene naturgemäß aufwendiger, da sie nur indirekt erbracht werden kann. p53 hat eine wesentliche Funktion darin, DNA-Schäden, z. b. durch UV-Strahlung zu erkennen und die Zelle vor Replikation dieser DNA z. B. in den programmierten Zelltod (Apoptose) zu führen. p53 wird daher auch als „Wächter des Genoms“

bezeichnet. Ein Funktionsverlust von p53 führt zu einer zunehmenden genomischen Instabilität, welche charakteristisch für fortgeschrittene Karzinome ist (2, 84). Weitere und für das kolorektale Karzinom spezifischere Suppressorgene sind das APC (***A***denomatosis ***P***olyposis ***C***oli)-Gen und die beim HNPCC-Syndrom häufig inaktivierten DNA-Reparaturgene hMLH1 und hMSH2 (31).

1.2.3 Mikrosatelliten-Instabilität als Resultat defizienter Reparaturgene

Mikrosatelliten bestehen aus hochrepetitven DNA-Sequenzen einfacher Wiederholungen von 1-6 Basenpaaren. Dinucleotidfolgen, z. B. „ACACACAC..“. kommen dabei häufiger vor als Tri- oder Tetranucleotidfolgen. Mikrosatellitensequenzen sind meistens in nicht-transkribierten Genabschnitten (Introns) lokalisiert. Die Anzahl der Wiederholungen ist hochpolymorph, d. h. zwei Allele eines Individuums unterscheiden sich mit großer Wahrscheinlichkeit hinsichtlich eines Mikrosatelliten. Dadurch sind Mikrosatelliten u. a. ausgezeichnete Marker, um den Weg definierter Allele über Generationen zu verfolgen und bilden gleichsam einen genetischen Fingerabdruck eines Individuums.

Während der DNA-Replikation kommt es zu Lesefehlern und damit zu Falscheinbauten von Nucleotiden. Besonders störanfällig hierfür sind hochrepetitive Sequenzen. Zur Korrektur dieser Lesefehler verfügt die Zelle über ein Reparatursystem aus Helikasen, die den DNA-Strang entwinden, Exonukleasen, welche falsch eingebaute Nucleotide entfernen und spezifischen Proteinen, die zur Erkennung der Replikationsfehler erforderlich sind. Komponenten dieses Proteinkomplexes werden u. a. durch die beim HNPCC-Syndrom in der Keimbahn mutierten Gene hMLH1 und hMSH2 codiert. Der Funktionsverlust dieser Gene durch somatische Inaktivierung des zweiten Allels führt zur Mikrosatelliten-Instabilität, und zwar vorzugsweise in kolorektalen Epithelzellen, worauf Tumoren mit einem sogenannten „Mutator-Phänotyp“ entstehen (12, 43, 48).

1.2.4 Cycline als Zellzyklus-regulierende Protoonkogene

Eine Vielzahl von Onkogenen und Tumorsuppressorgenen reguliert unmittelbar die Expression von Cyclinen, welche die Phasen des Zellzyklus, und damit für die Tumorentstehung so fundamentale Ereignisse wie DNA-Replikation und Zellteilung, kontrollieren. Die Wissenschaftler Hartwell, Nurse und Hunt, 2001 Nobelpreisträger für Medizin, legten den Grundstein für unser heutiges Verständnis von der Regulation des Zellzyklus. Während Hartwell und Nurse an Hefe-Mutanten Proteine isolierten, die für den geregelten Ablauf des Zellzyklus erforderlich sind (36, 65), wies Hunt an Seeigeleiern Proteine nach, deren Konzentrationen sich mit der jeweiligen Phase des Zellzyklus abrupt änderte, und bezeichnete 1983 diese oszillierenden Proteine als Cycline (28). Aufgrund der weitgehenden Konservierung dieser Proteine im Laufe der Evolution, konnten die gewonnenen Ergebnisse unmittelbar auf menschliche Zellsysteme übertragen werden. Dies hatte die Identifizierung weiterer Cyclin-Familien wie z. B. der G1-Phase spezifischen Cycline D (87) und E (32, 66, 71) zur Folge. Es zeigte sich, daß Cycline nicht nur in direktem Zusammenhang mit bekannten Onkogenen und Tumorsuppressorgenen stehen, sondern ihrerseits zelltransformierende Eigenschaften besitzen und damit selbst als dominante Onkogene wirken können (8, 33, 46).

Zu der Familie der Cycline treten eine Vielzahl Cyclin-abhängiger Proteinkinasen, die durch Phosphorylierung und Dephosphorylierung in der Lage sind, mit unterschiedlichen Cyclinen zu assoziieren und deren Aktivität zu steuern. Die Assoziation der Komplexe aus Cyclinen und ihrer katalytischen Partner wird durch Bindung Kinase-spezifischer Proteine (CKDIs) inhibiert. Es entsteht somit ein reich vernetztes, untereinander abhängiges Puffersystem, daß zum einen Signale von außen moduliert, als auch intern Rückmeldungen über einen regelrechten Ablauf des Zellzyklus aufnimmt, diese an Kontrollpunkten zusammenfaßt und weitere Schritte bis hin zur Zellteilung initiiert (73).

Nach erfolgter Mitose können Zellen entweder in einer Ruhe-Phase, G0-Phase, verbleiben, oder in die G1-Phase eintreten, in der die Entscheidung getroffen wird, ob die Zelle in die S-Phase eintritt, oder vor Einleitung der DNA-Replikation differenziert und/oder in den programmierten Zelltod, die Apoptose, übergeht. Ein

wichtiger Kontrollpunkt am Übergang zur S-Phase fällt zeitlich mit der Phosphorylierung des Retinoblastoma-Proteins (RB) zusammen und wurde als Restriktionspunkt bezeichnet (68, 110). Hat die Zelle diesen Punkt durchlaufen, übernimmt ein internes Kontrollsystem die Steuerung des übrigen Zellzyklus bis zu dessen Komplettierung, dem Abschluß der Mitose-Phase (Abb. 3).

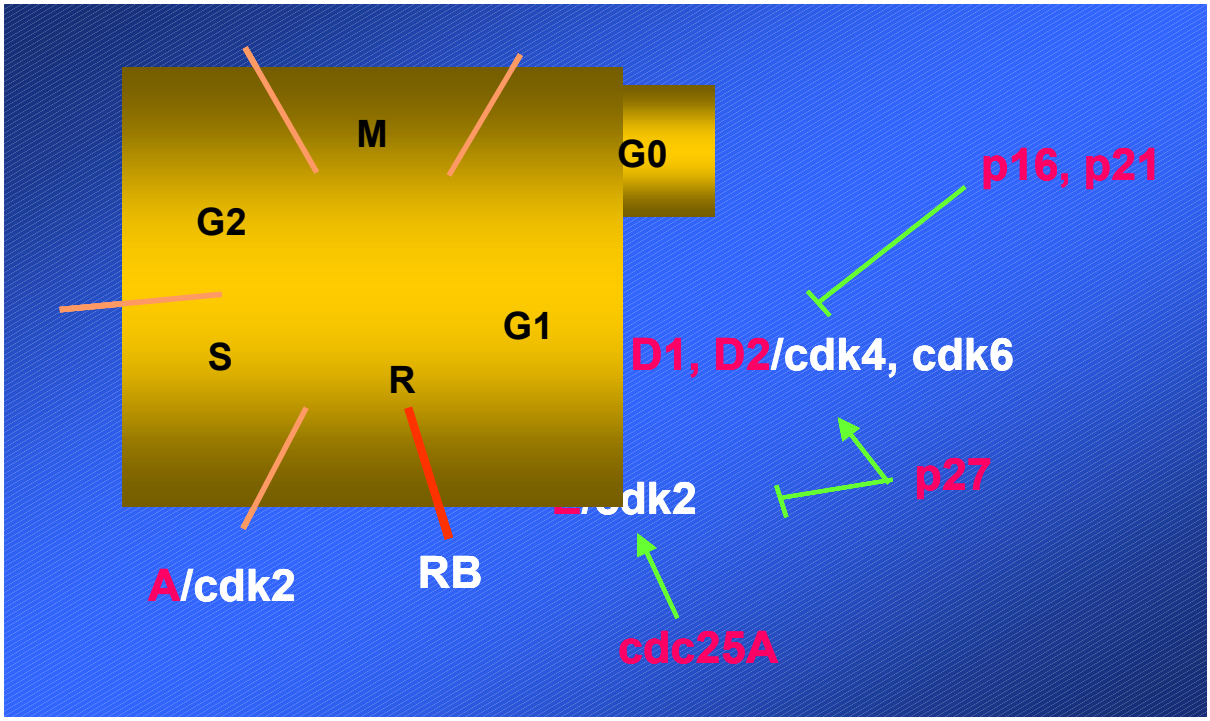

Abb. 3: Phasen des Zellzyklus, Restriktionspunkt (R) assoziiert mit der Phosphorylierung des Retinoblastoma-Proteins (RB) und dem Übergang der G1- in die S-Phase.

Die Phosphorylierung von RB ist das Ergebnis der Aktivierung G1-spezifischer Cyclin/Kinase-Komplexe und der Überwindung Kinase-abhängiger Inhibitoren (CDKIs). Geht man von einem gleichsam stöchiometrischen Gleichgewicht zwischen Inhibitoren, Cyclinen und Cyclin-abhängigen Kinasen (CDKs) aus, kann dies entweder durch Überexpression der Cycline, der Kinasen oder durch eine Verminderung inhibitorischer Proteine geschehen. Das Zusammenspiel und die zeitlich versetzte Aktivierung und Inaktivierung von Cyclin/Kinase-Komplexen, die letztlich in der Phosphorylierung von RB münden und die Zellteilung triggern, stellen

somit ein interagierendes Puffersystem während der G1-Phase dar (41, 59, 112). Die Aufgabe dieses Puffers ist es, von außen einwirkende Stimuli, wie Wachstumssignale oder Differenzierungssignale aufzufangen, gewebespezisch zu modulieren und für eine zeitlich geordnete Umsetzung dieser Botschaften zu sorgen. Die Fähigkeit zur klonalen Expansion ist eine unabdingbare Voraussetzung für die Proliferation maligner Tumoren. Zellteilungen müssen in rascher Folge durchlaufen werden und fehlerfrei ablaufen, was physiologischerweise an distinkten Kontrollpunkten des Zellzyklus überprüft wird. Für eine effektive DNA-Synthese muß z. B. ein hochspezialisiertes Proteinsystem zur Verfügung stehen und u. a. Histon-RNA exprimiert werden (57, 120). Ebenso ist auch der programmierte Zelltod ein aktiver, genetisch kontrollierter, zielgerichteter Vorgang, assoziiert u. a. mit der Spaltung des Chromatins, der Inaktivierung bestimmter Proteine, und somit basierend auf einer Kette von Gen-Induktionen und/oder -Inaktivierungen.

1.2.5 Aktuelles Konzept der molekularen Karzinogenese unter besonderer Berücksichtigung der Cycline

Die Umwandlung normaler Zellen zu einem malignen Tumor ist ein mehrstufiger Prozeß. Dieser beginnt mit einem, z. B. durch ein Karzinogen, ausgelösten Initialereignis, der Initiation, im Sinne einer Mutation oder einer epigenetischen Veränderung. Es folgt der Prozeß der Promotion unter der ggf. jahrelangen Einwirkung von Substanzen, die z. B. das im Kolonepithel vorliegende Gleichgewicht zwischen Proliferation und Differenzierung zugunsten einer gesteigerten Proliferation verschieben. Kennzeichnend für die Phase der Tumorpromotion ist außerdem ein Verlußt der Inhibition sog. Kontaktstellen ("gap-junctions"). Die Phase der Tumorpromotion ist teilweise noch reversibel. Sie entspricht klinisch dem Stadium der Kolonadenome. Zellen, die aus Kolonadenomen isoliert und kultiviert werden, sind immortalisiert, d. h. unbegrenzt teilungsfähig, besitzen aber noch nicht die Merkmale eines malignen Phänotyps.

Der Übergang zum Karzinom leitet die dritte Phase des Mehrstufenprozesses, die Tumorprogression, ein. An dieser Schnittstelle erhalten die Zellen Fähigkeiten, die

allen von Karzinomen abstammenden Zellen eigen sind. Dazu gehören u. a. das 3-dimensionale Wachstum in Agar, unabhängig von einem Haften auf Kulturschalen („anchorage independency“), und die Bildung von Tumoren, die sog. Tumorgenität, in der Nacktmaus. Im Laufe der fortschreitenden Progression erwerben die Zellen durch weitere genetische und/oder epigenetische Veränderungen die Fähigkeit zur Metastasierung, welche tierexperimentell z. B. durch i.v.-Injektion von Tumorzellen und Ausbildung von Lungen- und/oder Lebermetastasen überprüft werden kann (3).

In der Kolonkarzinogenese gehören die Mutationen von K-ras, der Verlust der APC-Funktion und die Mikrosatelliten-Instabilität zu den initialen Ereignissen, da diese bereits in aberranten Krypten nachzuweisen sind (72, 99). Der Schritt der Initiation kann im Tierexperiment durch Karzinogene wie N-methyl-N’-nitro-N-nitrosoguanidin (MNNG), welches eine spezifische ras-Mutation auslöst (78), oder durch sogenannte „knock out“-Mäuse, z. B. durch APC-defiziente Mäuse simuliert werden (60).

Als Tumorpromoter wirkt z. B. Phorbolester, ein Aktivator der Proteinkinase C (PKC), welcher eine Kaskade von Phosphorylierungs- und Dephosphorylierungsprozessen auslöst und schließlich Proliferationsgene wie c-jun und c-fos aktiviert. In ähnlicher Weise könnten in vivo Gallensäure in Verbindung mit Darmbakterien als natürliche Promoter der Kolonkarzinogenese wirken, da sie nachweislich intraluminär Diacethylglycerol (DAG) freisetzen, welches ebenfalls die PKC aktiviert (77).

Ein grundlegendes Konzept der molekularen Karzinogenese besteht darin, daß die Kooperation mehrerer aktivierter Onkogene und/oder der Verlust von Suppressorgenen notwendig ist, um einen malignen Phänotyp auszubilden. Membranständige Proteine, z. B. der Ras-Familie, könnten dadurch mit zytoplasmatischen Signalübermittlern (PKC) interagieren und die Expression nukleärer Transkriptionsfaktoren (c-jun, c-myc, Cycline) regulieren (111).

Ein weiterer Signalweg, welcher insbesondere für die kolorektale Karzinogenese von Bedeutung ist, wird durch Wnt-Proteine aktiviert. Im Mittelpunkt dieses Signalwegs steht das APC-Protein, welches den Ubiquitin-abhängigen Abbau von β-Catenin vermittelt (Abb. 4). Eine inaktivierende Mutation im APC-Gen, sei es als Keimbahnmutation bei der Polyposis coli oder als somatische Mutation in ca. 80%

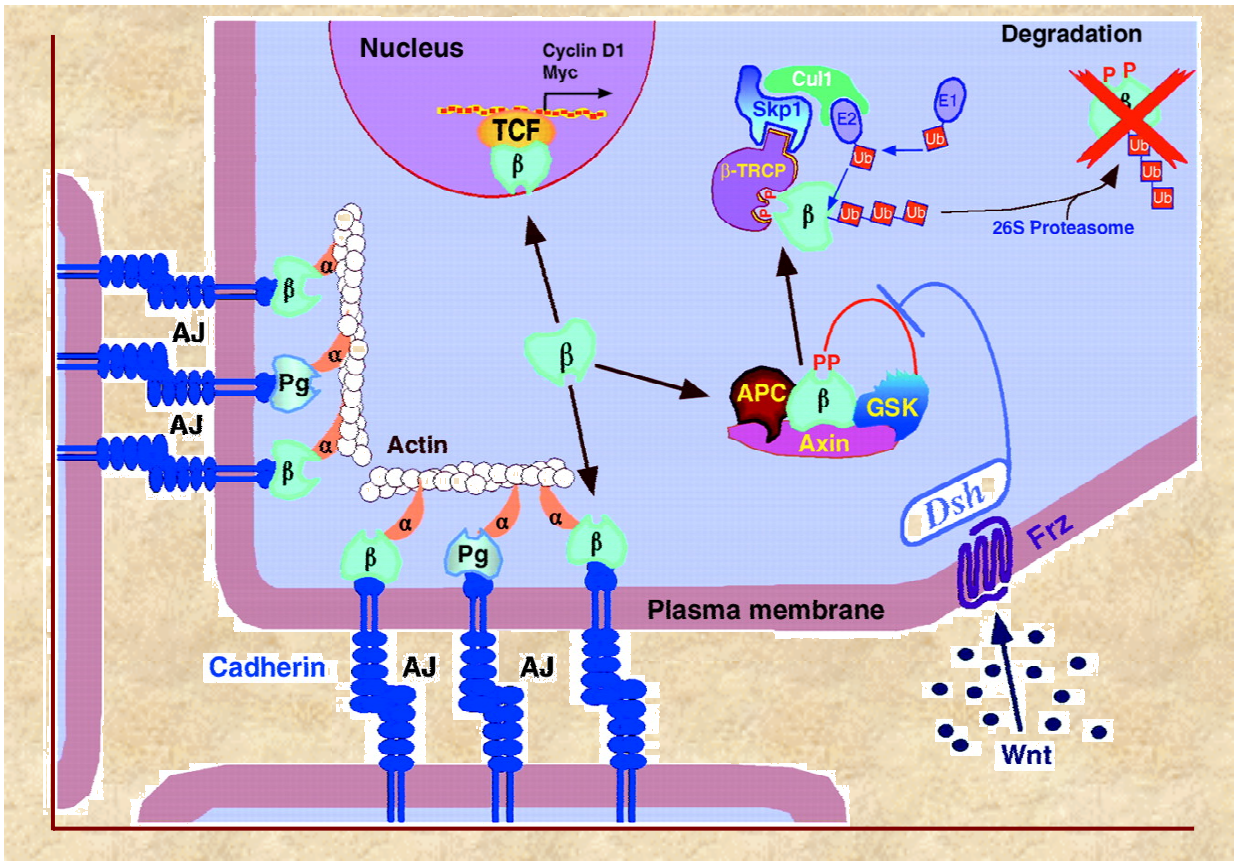

Abb. 4: Wnt-Signalweg mit nachfolgender Induktion von Cyclin D1 und c-myc; β, β-Catenin; modifiziert nach J. Zhurinsky et al. (122).

der sporadischen kolorektalen Karzinome, führt zu einer Akkumulation von β-Catenin im Zellkern. Hier assoziiert β-Catenin mit dem T-Zell-Faktor (TCF) wodurch u. a. die Transkription Zellzyklus-regulierender Proteine wie Cyclin D1 und c-myc induziert wird (29).

Die Vermutung, daß Zellzyklus-regulierende Proteine eine Bedeutung für die Entstehung menschlicher Tumoren haben könnten, beruhte zunächst auf der Identifizierung eines Gens, das in Nebenschilddrüsentumoren mittels Translokation unter die Kontrolle des Parathormon-Promoters gelangt, damit überexprimiert wird, und als PRAD1-Gen bezeichnet wurde (61). PRAD1 entspricht Cyclin D1, welches auch in Lymphomen transloziert ist (79). Amplifikationen und/oder eine erhöhte Expression von Cyclin D1 wurden in so unterschiedlichen Karzinomen wie Plattenepithel- (45) und Adenokarzinomen (5) des Ösophagus, Karzinome des HNO-

Bereiches (58), in Leberzellkarzinomen (118, 119), Mammakarzinomen (7, 50), Bronchialkarzinomen (117) und kolorektalen Karzinomen (4, 98) nachgewiesen. Die Hypothese, daß Cyclin D1 als ein mögliches Onkogen anzusehen ist wird weiterhin durch die Cyclin D1-abhängige Zelltransformation in vitro (8, 46, 55) sowie durch die in Cyclin D1-transgenen Tieren hervorgerufenen Mammakarzinome (105) und Leberzellkarzinome (21) unterstützt.

Im Gegensatz zu Cyclin D1 wurden die übrigen G1-Cycline weniger häufig in menschlichen Tumoren untersucht. Überexpressionen und/oder aberrante Proteinformen von Cyclin E wurden in Mamma- (47) und Kolonkarzinomen (98) beschrieben, wobei Amplifkationen dieser Gene in Kolonkarzinomen selten auftreten (51). Eine Cyclin E-Überexpression konnte mit Magen- (63), Ovarial- (97) und Mammakarzinomen (47) fortgeschrittener Stadien assoziiert werden. Cyclin E kooperiert mit Ha-Ras bei der malignen Transformation von Fibroblasten (33) und induziert als Transgen eine Brustdrüsenhyperplasie sowie Mammakarzinome (14). Für Bronchialkarzinomen ist Cyclin E ein unabhängiger Prognoseparameter (62).

Entsprechend der zentralen Bedeutung der G1-Phase in der Entscheidung zwischen Proliferation, Differenzierung und/oder Apoptose sind alle G1-Phase-Cycline sowie deren verwandte Gene geeignete Kandidaten eines Onkogens oder Tumorsuppressorgens (88). Es konnte gezeigt werden, daß im RB-Signalweg die Expression mindestens eines, häufig jedoch mehrerer Gene, in allen menschlichen Karzinomen verändert ist, und zwar durch unterschiedlichste Mechanismen wie Cyclin D1-Induktion, cdk4-Amplifikation oder p16-Mutation (81, 89, 96, 100).

Expressionsmuster von Cyclinen, ihrer Kinasen, vor allem jedoch von Inhibitoren der Cyclin/Kinase-Komplexe, allen voran p27, erwiesen sich als unabhängige Prognosefaktoren häufig vorkommender Karzinome, wie z. B. Kolon- (54), Mamma- (75), Prostata- (104) und Bronchialkarzinome (27).

2 Fragestellungen

Basierend auf den dargelegten Forschungsergebnissen wurden bezüglich intestinaler Tumoren folgende Fragen untersucht:

1. Wie häufig sind K-ras-Mutationen in Adenokarzinomen des Dünndarms? In Ösophagus- und Magenkarzinomen sind K-ras-Mutationen nur selten nachzuweisen, während sie in über 40% der Kolonkarzinome vorhanden sind. Aufgrund epidemiologischer Parallelen zwischen Dünndarm- und Kolontumoren wäre es von Interesse, ob in Dünndarmkarzinomen ein ähnliches Mutationsspektrum wie in Kolonkarzinomen vorliegt. Um dies zu überprüfen wurden paraffin-eingebettete Dünndarmkarzinome mittels PCR auf K-ras-Mutationen hin untersucht (Anlage 1).

2. Weisen primäre humane kolorektale Karzinome und deren Zellinien spezifische Expressionsprofile von Cyclin D1 und E auf? Cyclin D1 wirkt in vitro durch konstitutive Expression und in vivo als Transgen wie ein Onkogen. Eine Überexpression von Cyclin E wurde in einer Reihe von Tumoren mit einem fortgeschrittenen Stadium und einer ungünstigen Prognose assoziiert. Zur Untersuchung der Cyclin D1- und E-Expression in kolorektalen Tumoren wurden diese Cycline mittels Western- und Northern-Blot in primären kolorektalen Tumoren und deren Zellinien analysiert (Anlage 2).

3. Inwiefern unterscheiden sich K-ras transfizierte, tumorgene Klone intestinaler Epithelzellen (IEC18-R1/R10) von der nicht-transformierten, spontan immortalisierten Stammlinie (IEC18) hinsichtlich ihrer Cyclin D1-Expression? Werden durch konstitutive Überexpression von humanem Cyclin D1 IEC18-Zellen transformiert? Die IEC18-Zellinie sowie deren K-ras überexprimierende Klone wurden mittels Western- und Northern-Blot-Analyse auf ihre Cyclin D1-Expression hin untersucht. Zur Untersuchung einer möglicherweise transformierenden Wirkung von Cyclin D1 in intestinalen Zellen, wurde dessen Sequenz mittels eines retroviralen Vektors in der IEC18-Zellinie überexprimiert

und entsprechende Klone bezüglich ihres Wachstums und ihrer Tumorgenität charakterisiert (Anlage 3).

4. Besteht ein Zusammenhang zwischen einer erhöhten bzw. aberranten Cyclin E-Expression und dem Mutator-Phänotyp kolorektaler Karzinome? Ausgehend von der Beobachtung einer erhöhten und aberranten Cyclin E-Expression in einer Subpopulation kolorektaler Karzinome sowie in der HCT116-Zellinie, welche eine hMLH1-Mutation und damit einen Mutator-Phänotyp aufweist, führten wir eine Genotypisierung primärer kolorektaler Karzinome sowie korrespondierender normaler Kolonmukosa und eine Analyse der Proteinexpression von Cyclin E in diesen Proben durch (Anlage 4).

3 Patienten und Methodik

3.1 Patienten

Präparate von Dünndarmkarzinomen und kolorektalen Karzinomen, Kolonadenomen, korrespondierender normaler Mukosa stammen von Patienten, die an der Klinik für Allgemein-, Viszeral- und Gefäßchirurgie der Martin-Luther-Universität sowie der Klinik für Chirurgie der Columbia-Universität in New York operiert wurden. Gewebeproben instabiler Karzinome wurden zusätzlich aus dem Institut für Humangenetik der Universität Stettin/Polen untersucht. Nach Einwilligung der Patienten wurde das resezierte Gewebe unmittelbar in Flüssigstickstoff kryoasserviert und bei –80 °C gelagert bzw. in Formalin fixiert und in Paraffin eingebettet. Normale Schleimhaut wurde vom Resektionsrand, welcher dem Tumor am weitesten entfernt lag, durch Dissektion von der Submukosa isoliert.

3.2 Westernblot-Analyse

Gewebe und Zellinien wurden in einem Lysierpuffer (Anlage 3) durch wiederholtes Pipettieren homogenisiert, auf Eis für 30 Minuten lysiert und zentrifugiert. Die Proteinkonzentrationen der Proben wurden bestimmt und die Proteinlösungen in einem SDS-Puffer mit β-Mercaptoethanol erhitzt. Je Spur wurden 50 μg Proteinlysat auf einem 10% Polyacrylamid Gel elektrophoretisch in Laemmli-Puffer aufgetrennt. Danach erfolgte die Übertragung der Proteine mittels Elektrotransfer in Glycin-Puffer auf PVDF-Membranen. Die Membranen wurden zum Nachweis einer gleichmäßigen Ladung und Auftrennung der Proteine mit Ponceau-S gefärbt. Die Membranen wurden zum Nachweis der spezifischen Proteine mit Serum geblockt und mit den jeweiligen primären Antikörpern inkubiert. Nach Waschen mit PBS und 1% Tween erfolgte die Inkubation mit sekundären, Peroxidase-konjugierten Antikörpern und die Immunfluoreszenz mit Hilfe des ECL-Systems. Die Intensitäten der Banden wurden durch 2-D-Laserdensitometrie quantifiziert, und die Intensitäten

der Tumorexpression mit Intensitäten korrespondierender normaler Mukosa verglichen.

3.3 Immunhistochemie

Gefrierschnitte von normaler Mukosa und kolorektalen Tumoren wurden in Aceton fixiert und mit 1% H_2O_2 behandelt. Danach wurden die Präparate mit PBS gewaschen, mit 5% fettfreier Milch geblockt und mit dem primären Antikörper (Anlage 4) inkubiert. Die Schnitte wurden unter Anwendung der Avidin-Biotin-Methode mit biotinylierten sekundären Antikörpern inkubiert. Die Immunreaktion wurde durch DAB sichtbar gemacht. Als Negativkontrollen dienten Präparate, die statt des primären Antikörpers mit nicht-immunreaktiven Seren inkubiert wurden.

3.4 Northernblot-Analyse

Gesamt-RNA wurde nach der Methode von Chirgwin isoliert (Anlage 2). Zur Untersuchung der Primärtumoren wurde PolyA$^+$-RNA extrahiert. PolyA$^+$-RNA oder Gesamt-RNA wurden auf einem Formaldehyd-Agarose Gel aufgetrennt und auf Nitrozellulose übertragen. Die Blots wurden mittels der „Random-Prime-Extension"-Methode und markiertem dCTP sowie spezifischen DNA-Sonden inkubiert. Die GAPDH-Signale bzw. die Ethidiumbromid-Färbung der 18S- und 28S-rRNA wurden zur Kontrolle der Güte der RNA und zur Kontrolle gleicher Ladung herangezogen. Die Signale wurden mittels Autoradiographie detektiert und die Intensitäten mittels 2D-Laser-Densitometrie quantifiziert.

3.5 Mikrosatelliten-Analyse

Genomische DNA wurde aus Gewebeproben normaler Mukosa und kolorektaler Karzinome isoliert (Anlage 4). Aus den jeweiligen DNA-Proben wurden 5 Mikrosatelliten des von der International-HNPCC-Collaborative-Group empfohlenen Referenzpanels aus Mononucleotid- und Dinucleotidmarkern mittles fluoreszenz-markierter, flankierender Primer amplifiziert. Anschließend erfolgte die Genotypisierung auf einem denaturierenden Polyacrylamid-Gel mittels automatischer Fragmentanalyse. Traten bei Tumoren im Vegleich zu normaler Mukosa zusätzliche Peaks auf, wurden diese Marker als instabil bewertet (Abb. 7). Karzinome wurden als hochinstabil eingestuft, wenn mindestens 2 der 5 untersuchten Marker instabil waren (12).

3.6 Polymerase-Kettenreaktion (PCR)

Gesamt-DNA wurde aus fixiertem, in Pararaffin eingebettetem Gewebe isoliert (Anlage 1). Codon 12 von K-ras wurde mittels eines 5‘-Mismatch-Primers amplifiziert, der eine Restriktionsstelle für das Enzym Bst N1 enthielt. Im Falle einer Wildtyp-Sequenz konnte somit das Amplifikat durch Bst N1 verdaut werden, während im Falle einer Punktmutation in einem der beiden ersten Nukleotide von Codon 12 die Sequenz durch Bst N1 nicht erkannt wurde. Zur Kontrolle der Enzymfunktion wurde auch am 3‘-Ende ein Nucleotid substituiert. Der Nachweis des daraus resultierenden DNA-Längen-Polymorphismus erfolgte durch Auftrennen der PCR-Produkte auf einem 8% Polyacrylamid-Gel und Färben mittels Ethidiumbromid. Als Kontrollen dienten Zellinien mit bekannter Mutation im Codon 12 des K-ras-Gens bzw. mit entsprechenden Wildtyp-Sequenzen. Die Nachweisgrenze des Verfahrens wurde durch Verdünnungsreihen genomischer DNA der genannten Zellinien ermittelt und betrug mindestens 1:16.

4 Ergebnisse

4.1 K-ras-Mutationen in Dünndarmkarzinomen

K-ras-Mutationen sind am häufigsten in Pankreaskarzinomen nachzuweisen, wobei hier ähnlich wie beim kolorektalen Karzinom Codon 12-Mutationen vorherrschen. Im Ösophagus sowie im Magen hingegen spielen K-ras-Mutationen kaum eine Rolle. Hinsichtlich der Prävalenz von Mutationen im K-ras-Gen gleichen cholangiozelluläre Karzinome dem Pankreaskarzinom. Da Dünndarmkarzinome zwar deutlich seltener als kolorektale Karzinome auftreten, jedoch epidemiologisch im Hinblick auf mögliche karzinogene Faktoren kolorektalen Karzinomen ähnlich sind, ergab sich daraus die Frage der Häufigkeit von K-ras-Mutationen in Adenokarzinomen des Dünndarms (Anlage 1).

Wir amplifizierten hierzu DNA aus Dünndarmkarzinomen mittels der genannten Mismatch-Primer, die Restriktionsstellen für Bst N1 enthielten. In 10 von 17 Fällen, darunter bei Karzinomen, die bis zu 13 Jahren in Paraffin eingebettet waren, gelang die PCR. Acht der 10 untersuchten Dünndarmkarzinome zeigte eine Mutationen in K-ras, Codon 12, ein Anteil der über dem kolorektaler Karzinome lag.

4.2 Analyse von Cyclin D1 und Cyclin E in primären kolorektalen Karzinomen und deren Zellinien

Zur Klärung, ob eine vermehrte Expression von G1-Phase-Cyclinen mit der Kolonkarzinogenese einhergeht, wurden Kolontumoren so ausgewählt, daß sie die Dukes-Stadien A-C und unterschiedliche Differenzierungsgrade repräsentierten (Anlage 2). Die Westernblot-Analyse primärer Kolontumoren und korrespondierender normaler Kolonmukosa mittels eines polyklonalen anti-Cyclin D1-Antikörpers zeigte Proteinprodukte von 30 und 36 kD in allen untersuchten Proben. Fünf von 11 Karzinomen wiesen eine mindestens 2-fache Expressionserhöhung von Cyclin D1 gegenüber normaler Mukosa auf. Der monoklonale anti-Cyclin E-Antikörper

erkannte ein 51 kD-Protein. Daneben konnten jedoch Proteinformen von niedrigerem Molekulargewicht, und zwar bis zu 30 kD, nachgewiesen werden. In vier der 10 untersuchten Tumoren war die Cyclin E-Expression um mindestens das 2fache gegenüber normaler Mukosa erhöht. Eine Überexpression niedermolekularer Cyclin E-Proteinformen lag in Dukes B-Kolonkarzinomen und in einem gering differenzierten, metastasierten Ovarialkarzinom vor.

Aus Primärgewebe isolierte mRNA wurde daraufhin mit Cyclin D1- und Cyclin E-cDNA-Sonden hybridisiert. Kolonkarzinome und normale Schleimhaut wiesen überwiegend ein Cyclin D1-Transkript von 4.5 kb und in geringerem Maße auch ein Transkript von 1.7 kb auf. Die densitometrische Auswertung der Expression des 4.5 kb-Transkripts ergab, daß die Expression von Cyclin D1 in 6 der 8 untersuchten Tumoren im Vergleich zu normaler Schleimhaut um das 1.7- bis 2.4fache erhöht war. Zwei Proben, ein Karzinom im Stadium Dukes A und ein Dukes B-Karzinom wiesen eine mindestens zweifach erhöhte Cyclin D1-Expression auf. Die Hybridisierung mit Cyclin E-cDNA zeigte ein einziges Transkript von 2.2 kb, welches in 6 der 8 Tumoren signifikant erhöht war.

Aufgrund vorausgegangener Nachweise von Amplifikationen von Cyclin D1 in Ösophaguskarzinomen wurden 18 Kolonkarzinome und 15 Proben normaler Kolonmukosa auf Amplifikationen von Cyclin D1 oder Cyclin E hin untersucht. Genomische DNA wurde für die Untersuchungen auf Cyclin D1 mit EcoR1 verdaut, was in DNA-Fragmenten von 4.0, 2.2 und 2.0 kb resultierte. Die Analyse von Cyclin E basierte auf der Restriktion mit BamH1 und dem Nachweis von 12 kb und 4.0 kb Fragmenten. Vergleichbare Ladung und vollständiger Verdau wurden mittels Ethidiumbromid-Färbung kontrolliert. In keinem der untersuchten Karzinome konnte eine signifikante Veränderungen der Cyclin D1- oder Cyclin E-Fragmente nachgewiesen werden.

Um weiter auszuschließen, daß die in Kolonkarzinomen beobachtete Erhöhung der Cyclin D1- und Cyclin E-Expression lediglich Ausdruck einer gesteigerten Zellproliferation ist, wurden Kolonkarzinomzellen unterschiedlicher Herkunft und Wachstumseigenschaften hinsichtlich ihrer Cyclin D1- und Cyclin E-Expression untersucht.

Interessant waren hier Expressionsunterschiede in Klonen der SW480-Zellinie. SW480-R2 stellt einen Klon mit hoher maligner Potenz und niedriger Verdopplungszeit (15 Stunden) dar, während SW480-E8 eine epitheliale Morphologie, eine niedrigere Wachstumsrate (32 Stunden Verdopplungszeit) und ein geringeres Maß an Aneuploidie und Tumorgenität aufweist. Die Cyclin D1-mRNA-Spiegel waren hier somit invers zur Tumorgenität korreliert (Anlage 2). Ähnlich wie in Primärgeweben war die mRNA-Expression von Cyclin E insgsamt niedriger als die Cyclin D1-Expression und erforderte eine längere, bis zu einer Woche dauernden Autoradiographie. Dennoch konnte in allen untersuchten Zellinien ein Cyclin E-Transkript von 2.2 kb nachgewiesen werden. Auch die mRNA-Expression von Cyclin E folgte nicht unmittelbar der proliferativen Aktivität und Tumorgenität der Zellinien. Auffallend war vor allem die ungewöhnlich hohe Cyclin E-Expression in HCT116 Zellen. Diese Zellen stammen von einem Patienten mit einem HNPCC-Syndrom ab und weisen einen homozygoten Verlust des hMLH1-Gens auf.

Um zu überprüfen, ob die Cyclin D1- und Cyclin E-Expression auf mRNA-Ebene mit einer vergleichbaren Proteinexpression einhergeht, wurden Zellinien kolorektaler Karzinome und Subpopulationen dieser Zellinien hinsichtlich ihrer Cyclin D1- und E-Proteinexpression untersucht. Die Inkubation mit einem Cyclin D1-Antikörper zeigte, daß analog zur mRNA-Expression die höchsten Cyclin D1-Proteinspiegel in den SW480-E8-Zellen nachzuweisen waren. Es bestand jedoch nicht in allen Zellinien eine strikte Korrelation zwischen mRNA- und Proteinexpression. Bezüglich des Cyclin E-Proteins war die HCT116-Linie mit einer entsprechend der mRNA-Spiegel hohen Expression am auffälligsten. Dennoch konnte auch für Cyclin E keine vollständige Übereinstimmung zwischen Protein- und mRNA-Spiegel gefunden werden. Zu den rein quantitativen Unterschieden traten auch qualitative Merkmale. Drei der untersuchten Zellinien, einschließlich HCT116, exprimierten überwiegend Cyclin E-Proteine niedrigerer molekularer Masse (39-44 kD) anstelle des in den übrigen Zellinien vorkommenden 51 kD-Proteins.

4.3 Analyse der Cyclin D1-Expression in K-ras-transformierten IEC18-Klonen und Überexpression von Cyclin D1 in der IEC18-Zellinie

Da bislang zur Untersuchung der Kolonkarzinogenese keine immortalisierten menschlichen Kolonepithelzellen zur Verfügung stehen, wurden spontan immortalisierte intestinale Rattenepithelzellen (IEC18) und K-ras-transformierte, tumorgene Klone dieser Zellinie kultiviert. Die Cyclin D1-mRNA-Expression war in K-ras-transformierten Klonen der IEC18-Zellinie im Vergleich zur Ausgangslinie deutlich erhöht (Abb. 5, Anlage 3).

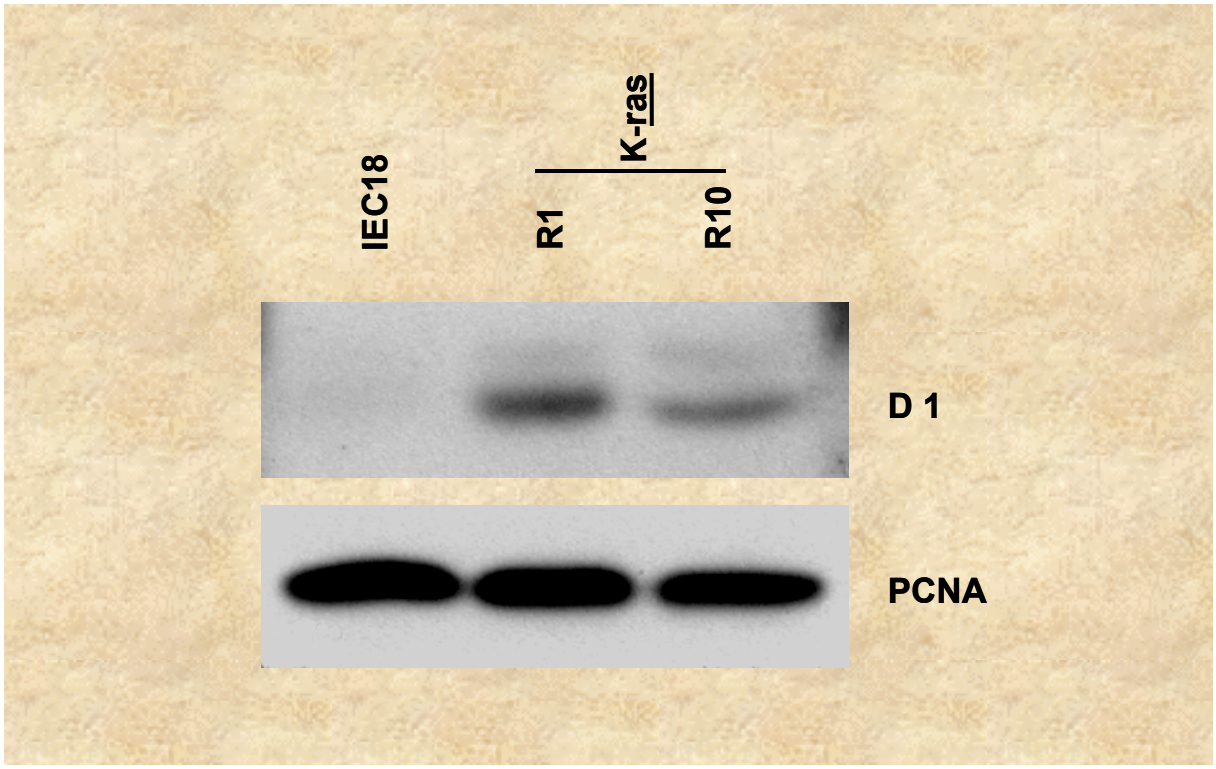

Abb. 5: Analyse der Cyclin D1-Expression in einer intestinalen Epithelzellinie (IEC18) und deren Klone mit konstitutiver K-ras-Expression (IEC18-R1, IEC18-R10).

Mittels eines retroviralen Vektors wurde daraufhin Cyclin D1-cDNA in IEC18-Zellen überexprimiert und Klone mit über 20-facher Cyclin D1-Proteinexpression im Vergleich zur Vektorkontrolle isoliert und weiter charakterisiert (Abb. 6). Die deutlich erhöhte Cyclin D1-Expression der isolierten Klone hatte weder einen Einfluß auf die

Morphologie, noch auf das Wachstum der Zellen in Monolayer-Kulturen und in Weichagar. Im Nacktmaus-Modell zeigte sich selbst nach subkutaner Injektion von je 10^7 Zellen Cyclin D1-überexprimierender IEC18-Klone in 4 Wochen alte Mäuse und einer Beobachtungszeit von 3 Monaten keine Tumorbildung. Als Positivkontrolle diente eine Kolonkarzinom-Zellinie (HT 29) mit deutlicher Koloniebildung in Weichagar und Tumorgenität in der Nacktmaus (Anlage 3)

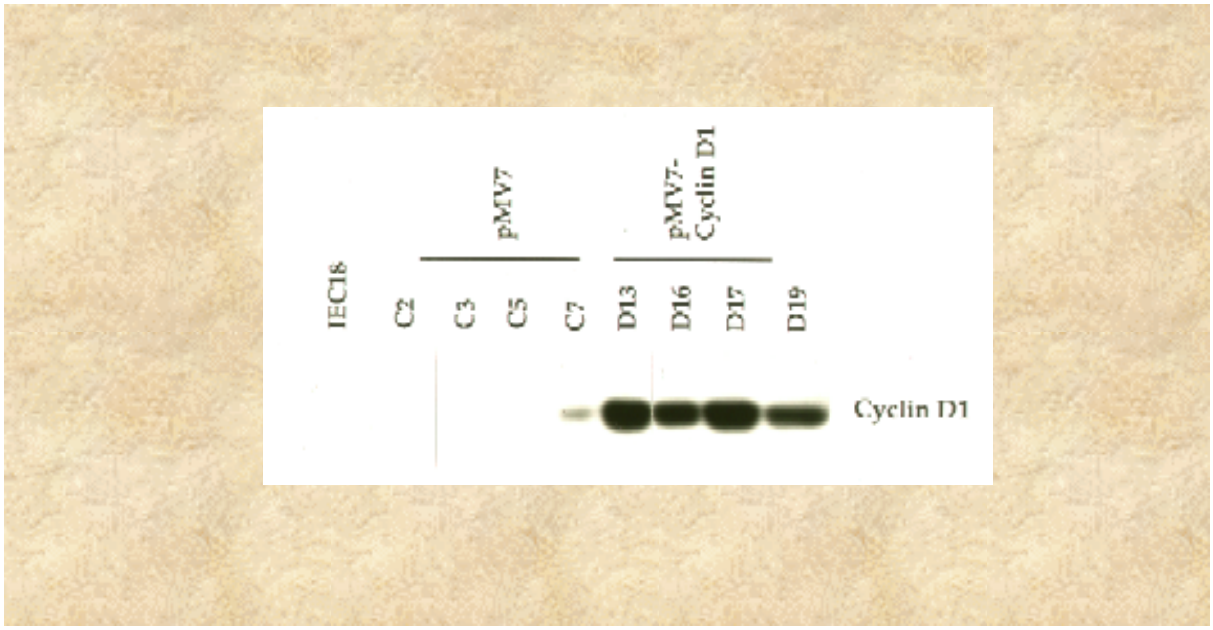

Abb. 6: Überexpression von Cyclin D1 in der IEC18-Zellinie mittels retroviralem Vektor (pMV7), Westernblot-Analyse überexprimierender Klone (D13, D16, D17, D19) und der Kontrollklone (C2, C3, C5, C7) sowie der Stammlinie IEC 18.

4.4 Die Assoziation von Mikrosatelliten-Instabilität und erhöhter bzw. aberranter Cyclin E-Expression in kolorektalen Karzinomen

Ausgehend von der Beobachtung einer relativ hohen Cyclin E mRNA- und Protein-Expression in Kolonkarzinomen im Vergleich zu Cyclin D1 und der qualitativen Veränderungen in der Proteinexpression von Cyclin E in Kolonkarzinomzellinien und in primären Kolontumoren konzentrierten sich die folgenden Untersuchungen auf die Regulation von Cyclin E innnerhalb eines erweiterten Patientenkollektivs. Aufgrund der relativen Cyclin E-Überexpression einer MSI-positiven und hMLH1-mutierten Zellinie (HCT116) analysierten wir unter Ermittlung des MSI-Status die Expression von Cyclin E in MSI-positiven und MSI–negativen Tumoren. Dabei wurden vorrangig Westernblot-Analysen angewandt, da post-translationale Regulationsmechanismen für die Expression von Zellzyklusgenen von Bedeutung sind und insbesondere bei Cyclin E-Proteinen unterschiedlicher molarer Masse nachgewiesen werden können (Anlage 4).

Bei 100 Patienten wurde nach Isolierung genomischer DNA aus normaler Kolonmukosa und Karzinomgewebe eine Mikrosatelliten-Analyse durchgeführt. Hohe Mikrosatelliten-Instabilität (MSI), also Instabilität in mindestens 2 von 5 untersuchten Markern, lag bei 13 der 100 Patienten vor. MSI zeigte sich am häufigsten in Mononucleotidrepeats. Diese waren bei allen Patienten mit MSI informativ. Niedrige Instabilität, d. h. Veränderungen in nur einem Marker, wurde in keinem der Fälle beobachtet. Vergleicht man Tumorstadien, Tumorlokalisation, Differenzierungsgrad und Alter so ergeben sich in den beiden Gruppen MSI-positiver und MSI-negativer Patienten keine signifikanten Unterschiede. Ein Beispiel der Mikrosatelliten-Analyse eines MSI-positiven Tumors ist in Abb. 7 dargestellt.

Abb. 7: Ergebnis einer automatisierten Genotypisierung eines kolorektalen Karzinoms (T) und korrespondierender normaler Mukosa (N) mittels fluoreszenz-markierter Primer für repetitive Mono- (Bat25, Bat26) und Dinucleotidsequenzen (D2S123, D5S346, D17S250)

Gleichzeitig zur Genotypisierung erfolgte die Untersuchung der Cyclin E-Proteinexpression mittels Westernblot-Analyse in Tumorgewebe und normaler Kolonmukosa. Ein monoklonaler anti-Cyclin E-Antikörper erkannte in der normalen Mukosa und in Karzinomen überwiegend Cyclin E-Proteine von 51 kD und 44 kD. Die Expressionsgrade der normalen Mukosa waren innerhalb einer Analyse weitgehend identisch.

Cyclin E-Proteine waren in 38 der 100 Proben um mindestens das 2-fache erhöht. Die densitometrische Auswertung zeigte, daß in einigen Fällen eine bis zu 20-fache Überexpression von Cyclin E, verglichen mit korrespondierender normaler Mukosa, vorlag (Abb. 8). Interessant war, daß es sich hierbei um Karzinome früher Stadien (Dukes A und B) handelte. Abb. 9 gibt die Verteilung der Tumorstadien in Bezug auf die Cyclin E-Überexpression wider. Cyclin E war in keinem der untersuchten Adenome erhöht, während innerhalb der Karzinom-Stadien keine signifikanten Unterschiede in der Cyclin E-Überexpression vorlagen. Vergleicht man das Cyclin E-Expressionsprofil mit den Differenzierungsgraden, so ergab sich ebenfalls kein

statistisch signifkanter Zusammenhang. Analog zu der Stadienverteilung traten hohe Cyclin E-Expressionen sowohl in hoch- als auch in gering differenzierten Karzinomen auf.

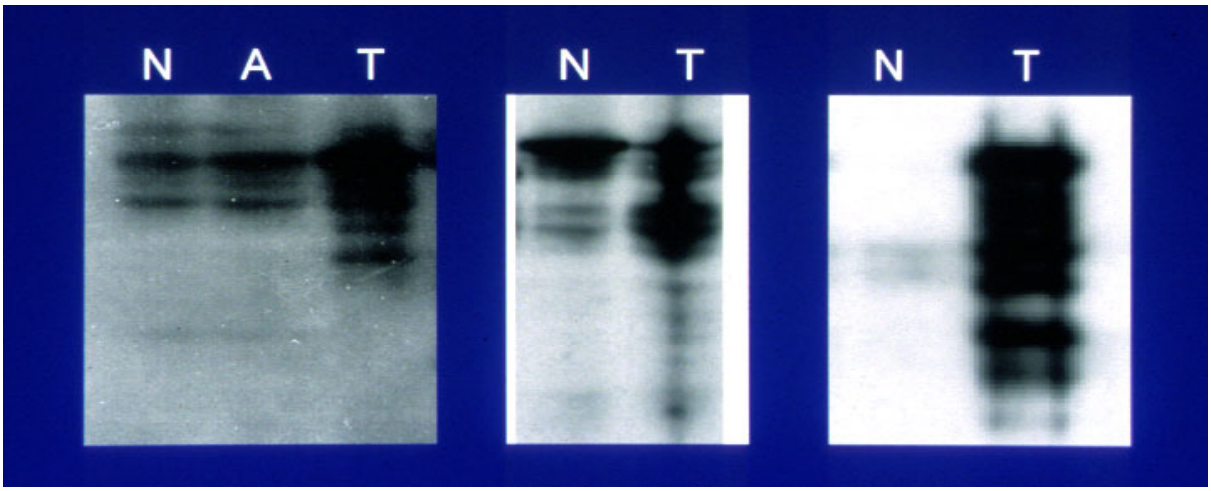

Abb. 8: Cyclin E-Proteinexpression in normaler Kolonmukosa (N), einem Kolonadenom (A) und in Kolonkarzinomen (T) des Stadiums Dukes B

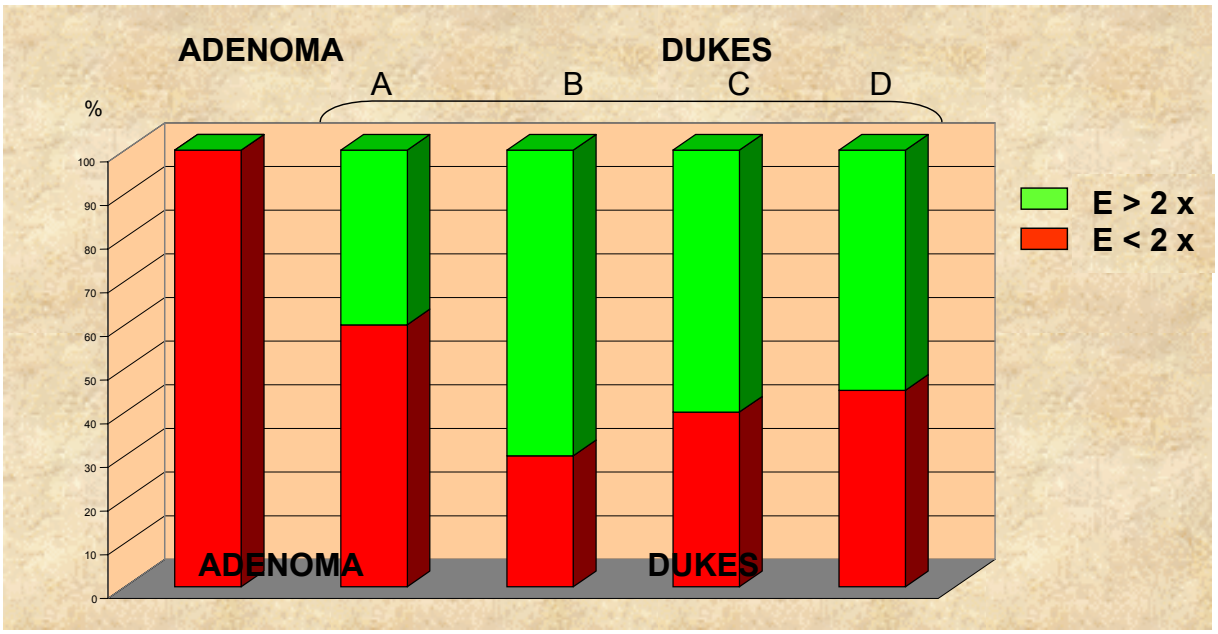

Abb. 9: Verteilung der Cyclin E-Überexpression in unterschiedlichen Stadien kolorektaler Tumoren

Auffallend bei der Auswertung der Cyclin E-überexprimierenden Tumoren war, daß eine Subpopulation von Tumoren erhöhter Cyclin E-Proteinexpression nicht nur die dominierenden Proteinformen von 51 kD und 44 kD, sondern auch schneller migrierende Cyclin E Proteine von 39 kD und darunter exprimierten. Interessanterweise handelte es sich dabei signifikant häufig ($p<0.001$) um Karzinome mit hoher Mikrosatelliteninstabiliät. Ein ähnlicher Zusammenhang konnte bereits durch eine rein quantitative Analyse der Cyclin E-Expression hergestellt werden. Cyclin E-überexprimierende Kolonkarzinome waren überzufällig häufig ($p<0.001$) MSI-positiv.

Um auszuschließen, daß die in der Westernblot-Analyse auftretende Überexpression von Cyclin E auf eine Kontamination mit nicht-epithelialem Gewebe, z. B. der bei HNPCC-assoziierten Karzinomen beschriebenen Lymphozyten-infiltration, zurückzuführen war, wurden normale Mukosa, Tumoren mit im Vergleich zu Mukosa deutlich erhöhter Cyclin E-Expression immunhistochemisch mittels Cyclin E-Antikörper untersucht. Dabei war die Expression von Cyclin E selbst in dem Tumor mit dem höchsten Cyclin E-Spiegel stets auf den Zellkern der Karzinomzellen lokalisiert. Stromazellen exprimierten kein Cyclin E. Normale Mukosa zeigte eine schwache Färbung in einzelnen Zellen an der Basis der Krypten. In allen analysierten Schnitten war die Cyclin E-Expression den Ergebnissen der Western-blots vergleichbar, wobei im Falle normaler Kolonmukosa die Westernblot-Analyse im Vergleich zur Immunhistochemie die sensitivere Nachweismethode darstellte.

Um weiter der Frage nachzugehen, ob die beobachtete Veränderung der Cyclin E-Expression in kolorektalen Tumoren verglichen mit korrespondierender Mukosa auf eine erhöhte Transkription und/oder alternatives Spleißen zurückzuführen war, wurde aus einer Serie von 20 Proben kolorektaler Karzinome und normaler Mukosa RNA isoliert, eine cDNA hergestellt und ein Cyclin E-Fragment amplifiziert. Zusätzlich wurde das kürzlich identifizierte Cyclin E-Homolog, Cyclin E2, in gleicher Weise untersucht. Die Primerpaare umfaßten dabei jeweils die zentrale Region des Cyclin E- und Cyclin E2-Gens. Die Analyse zeigte, daß in keiner der Proben Extrabanden im Sinne von Spleißvarianten amplifiziert wurden.

5 Diskussion

Die Bedeutung der Cycline für die Karzinomentstehung basiert auf der Beobachtung chromosomaler Aberrationen, wie Rearrangements, einhergehend mit erhöhter mRNA-Expression bei so seltenen Tumoren wie Nebenschilddrüsentumoren (61) und zentrozytischen Non-Hodgkin-Lymphomen (79). Bei einer Anzahl häufiger vorkommender Karzinome werden Amplifkationen der 11q13-Region nachgewiesen (85). Amplifikationen sind bei Kolonkarzinomen sowohl im Cyclin D1- als auch im Cyclin E-Gen selten (51), was durch die Ergebnisse der Southernblot-Analyse primärer Kolonkarzinome und Kolonkarzinom-Zellinien bestätigt wird.

Als weitere Ursachen einer erhöhten Expression könnte die für Cyclin D1 beschriebene Stabilisierung der mRNA in Betracht kommen (24). Denkbar wären auch Cyclin D1-Spleißvarianten mit einer erhöhten Stabilität (40, 118). Da wir jedoch weder im Northernblot noch in der RT-PCR Hinweise auf Spleißvarianten von Cyclin D1 oder Cyclin E fanden, könnte die erhöhte mRNA- und Proteinexpression von Cyclin D1 am ehesten Ausdruck einer Geninduktion, z. B. durch aktivierte Protoonkogene wie K-ras (11) und/oder die Inaktivierung des APC-Gens (29, 101) sein, während für die erhöhte und aberrante Cyclin E-Proteinexpression maßgeblich post-translationale Mechanismen verantwortlich zu sein scheinen (74, 93).

APC-Verlust und K-ras-Mutationen sind frühe Ereignisse in der Karzinogenese kolorektaler Karzinome (103). Dies erklärt, daß im Gegensatz zu Cyclin E, Überexpressionen von Cyclin D1 bereits in Vorstufen (Adenomen) kolorektaler Karzinome beobachtet werden können (4). Berücksichtigt man die hohe Prävalenz der genannten Mutationen, einschließlich der selten vorkommenden Dünndarmkarzinome, so ergibt sich daraus eine mögliche Rolle von Cyclin D1 in der Initiation von Karzinomen des Intestinaltraktes.

Wie unsere Studien an Zellinien unterschiedlicher Proliferationsraten zeigten, spiegelt dabei die Expression von Cyclin D1 nicht einfach eine erhöhte Proliferation wider, sondern könnte durch Deregulation eines geordneten Ablaufs des Zellzyklus zu Störungen in der DNA-Replikation, bzw. -Reparatur und nachfolgenden Zellteilung

führen. Interessanterweise zeigen Zellen mit konstitutiver Überexpression von Cyclin D1 zwar eine Verkürzung der G1-Phase, aber eine kompensatorische Verlängerung nachfolgender Abschnitte des Zellzyklus (35). Cyclin D1- und Cyclin E-überexprimierende Zellen entwickeln eine zunehmende genomische Instabilität im Sinne von Amplifikationen (121) respektive einer Polyploidie (94). Cycline könnten somit den Prozeß der malignen Transformation aktiv vorantreiben.

Höhere Konzentrationen des Cyclin E-Proteins reflektieren nicht immer deren mRNA-Spiegel. Insbesondere weisen Kolonkarzinome mit besonders hohen Cyclin E-Proteinspiegel keine entsprechende Geninduktion auf. Zusätzliche Banden in der Cyclin E-Proteinexpression sind Ausdruck einer allgemein gesteigerten Cyclin E-Expression, da diese Banden gelegentlich auch schwächer oder kaum detektierbar in Proben normaler Mukosa vorkommen. Denkbar wäre eine Stabilisierung des Proteins durch verminderten Abbau. Cyclin E wird u. a. über das Ubiquitin-System abgebaut (20, 93). Die einzelnen Komponenten dieses Systems sind noch nicht vollständig untersucht. Dabei scheinen Proteine der Cullin-Familie eine wichtige Rolle beim Abbau von Cyclin E zu spielen (91, 114). Es wäre denkbar, daß sowohl Mutationen im Cyclin E-Gen als auch in Komponenten des Ubiquitin-vermittelten Abbaus zu einer Stabilisierung und Überexpression des Cyclin E-Proteins in kolorektalen Karzinomen führen (116). Da in humanen Tumoren selten andere Cyclin E-Mutationen als Amplifikationen nachgewiesen wurden (23), scheint erstere Annahme unwahrscheinlich und wurde durch uns nicht näher untersucht. In diesem Zusammenhang ist es von Bedeutung, daß nachgewiesen werden konnte, daß die aberranten Cyclin E-Proteine Zwischenstufen eines nicht durch Ubiquitin vermittelten Proteinabbaus darstellen und hohe Kinase-Aktivität besitzen (37, 74), also biologisch relevant sind. Der Abbau von Cyclin E und dessen mögliche Veränderungen während der Kolonkarzinogenese ist jedoch nicht völlig geklärt.

Im Gegensatz zu Cyclin D1 konnte für Cyclin E keine Überexpression in Kolonadenomen nachgewiesen werden. Cyclin E scheint somit in unmittelbarem Zusammenhang mit der malignen Transformation epithelialer Zellen zu stehen, jedoch für die Progression von Karzinomen keine Bedeutung zu haben, da der Anteil Cyclin E-überexprimierender Karzinome über alle Stadien konstant bleibt.

MSI ist ein Charakteristikum einer Subpopulation von kolorektalen Karzinomen, die offensichtlich einen spezifischen Weg der Karzinogenese beschreiten. Diese Karzinome vom Mutator-Phänotyp machen etwa 10% aller kolorektalen Karzinome aus und haben gemeinsame klinisch-pathologische Eigenschaften. Unsere Analysen bestätigen, daß Mononukleotid-Marker besonders informativ sind (38, 42), und daß durch die Verwendung von zwei Mononukleotid-Markern alle hochinstabilen Karzinome identifiziert werden können. Die Erweiterung des Panels auf 10 und mehr Marker dürfte die Ausbeute nur noch geringfügig erhöhen, so daß wir auf die Genotypisierung weiterer Loci verzichtet haben.

Die beobachtete Assoziation zwischen einer Cyclin E-Überexpression, dem Auftreten aberranter Cyclin E-Proteine und einer Mikrostelliten-Instabilität ist ein weiterer Hinweis dafür, daß MSI-positive Kolonkarzinome nicht nur klinisch und histo-morphologisch eine Untereinheit bilden, sondern auch spezifische genetische Merkmale besitzen. Da MSI-positive Kolonkarzinome häufig Frameshift-Mutationen im TGFß-Typ II-Rezeptor aufweisen (102), könnte diese Inhibition des TGFß-Signalweges über eine verminderte Expression des Cyclin E-Suppressorproteins p27 zu einer Stabilisierung von Cyclin E führen. Die näheren Mechanismen, die den Ubiquitin-abhängigen sowie -unabhängigen Abbau von Cyclin E bewirken, sind jedoch nicht vollständig bekannt. Die Möglichkeit, daß die bei MSI-positiven Tumoren um das 1000fache erhöhte Mutationsfrequenz zu Mutationen im Cyclin E-Gen führt, und diese eine Stabilisierung des Proteins nachsichziehen, ist aufgrund seltener Mutationen im Cyclin E-Gen unwahrscheinlich (70).

Da wie eingangs erwähnt die Überexpression von Cyclinen die Deregulation nachgeschalteter Abläufe im Zellzyklus stören kann, wäre es auch denkbar, daß eine Cyclin E-Überexpression einen vorzeitigen Start der DNA-Replikation bewirkt, bevor die Zelle die notwendigen Vorbereitungen hierzu getroffen hat und dadurch den Mutator-Phänotyp unterstützt. In ähnlicher Weise kommt es durch Mutationen des Suppressorgens p53 zu einem Kontrollverlust der G1-Phase, was zu genomischer Instabilität führt (39). Die durch p53 verursachte genomische Instabilität ist nicht das Initialereignis der Tumorgenese, sondern ist mit einer Beschleunigung der Tumorprogression assoziiert, möglicherweise dadurch, indem sie die Rate weiterer Protoonkogen-Aktivierungen und/oder Inaktivierungen von Suppressorgenen

steigert. Parallel hierzu wäre es denkbar, daß Cyclin E im Gegensatz zu Cyclin D1 nicht für die Initiation und Promotion von Kolontumoren maßgebend ist, daher in Kolonadenomen nicht überexprimiert ist, hingegen eher eine Rolle bei der malignen Transformation und der zunehmenden genomischen Instabilität, z. B. der Mikrosatelliteninstabilität, spielt. Zur Unterstützung dieser Hypothese wäre es sinnvoll, Zellsysteme mit konstitutiver Cyclin E-Expression hinsichtlich ihrer DNA-Reparaturmechanismen und genomischen Instabilität weiter zu untersuchen.

Inwieweit können unsere Kenntnisse zur Zellzyklusregulation kolorektaler Karzinome klinisch genutzt werden? Cycline, Cyclin-abhängige Kinasen und ihre Suppressorproteine stehen physiologischerweise in einem stöchiometrischen Gleichgewicht zueinander und stellen gleichsam ein Puffersystem dar, das dazu dient, von außen auf die Zelle einwirkende Signale (Wachstumsfaktoren, Kontaktinhibition, etc.) zu modulieren und einen geordneten Ablauf des Zellzyklus zu gewährleisten. Kommt es nun im Laufe der Karzinogenese zu einer Deregulation einzelner Komponenten dieses Systems, müssen die übrigen Gegenspieler darauf eingestellt werden, damit die Zelle überlebensfähig bleibt. Die Zelle wird dadurch von der starken Veränderung in der Expression einzelner Gene abhängig („gene addiction“) und gegenüber einer gezielten Gegenregulation, z. B. mittels anti-sense-Strategien, verwundbar ("gene sensitivity") (112). Die Identifizierung von Subpopulationen kolorektaler Tumoren mit einer auffallend hohen Expression einzelner Zellzyklusgene könnte daher sowohl in der Chemoprävention (Cyclin D1) als auch in der Diagnostik und Gentherapie früher Stadien (Cyclin E) genutzt werden. Genchip-Technologien und Proteomforschung könnten es ermöglichen, Expressionsprofile abhängiger Zellzyklusgene routinemäßig zu analysieren (67). Die daraus gewonnenen Daten könnten im Rahmen multimodaler Behandlungskonzepte kolorektaler Karzinome Grundlage individuell adaptierter Therapieentscheidungen sein (92).

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7 Originalarbeiten

Frequent K-ras Mutations in Small Bowel Adenocarcinomas

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Digestive Diseases and Sciences, Vol. 41, No. 1 (January 1996), pp. 115 – 118

Expression of Cyclins D1 and E in Human Colon Adenocarcinomas

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Increased Expression of Cyclin D1 and the RB Tumor Suppressor Gene in c-K-ras Transformed Rat Enterocytes

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Overexpression of Cyclin E Protein is Closely Related to the Mutator Phenotype of Colorectal Carcinoma

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VII. 1

Frequent K-ras Mutations in Small Bowel Adenocarcinomas

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Frequent K-*ras* Mutations in Small Bowel Adenocarcinomas

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The reasons for the relatively rare occurrence of small bowel adenocarcinomas when compared to the high frequency of colonic adenocarcinomas are unknown. Activating mutations in the K-*ras* oncogene occur in about 40% of colonic adenocarcinomas, possibly reflecting the consequences of carcinogenic exposure. To study whether the low incidence of small bowel adenocarcinomas might be due to the absence of activation of cellular oncogenes in small bowel adenocarcinomas, we examined the frequency of K-*ras* mutations in small bowel adenocarcinomas. K-*ras* mutations were determined using a polymerase chain reaction (PCR) -based method to detect codon 12 mutations by restriction fragment length polymorphism. PCR amplification was successful in six of nine small bowel adenocarcinoma samples, and revealed point mutations of K-*ras* at codon 12 in five of these six cases. We conclude that the small bowel might be exposed to carcinogens similar to those responsible for colorectal cancer, but may have developed protective mechanisms against cancer formation.

KEY WORDS: small bowel neoplasms; K-*ras* mutations; intestinal carcinogenesis.

The prevalence of adenocarcinoma of the small bowel is about 100-fold less than that of colorectal carcinoma (1–3). However, small intestinal adenocarcinomas also share with colon cancer epidemiological features such as an increased incidence in Western nations (4), an association with cholecystectomy and alcohol consumption (5), and a relationship to fat and protein intake (6). Small intestinal adenomatous polyps are precursor lesions for adenocarcinomas, and some families with familial adenomatous polyposis are at increased risk for small intestinal malignancy (7). These studies support the hypothesis that the low occurrence of small intestinal cancer might therefore be due to cancer protective mechanisms of the small intestinal mucosa rather than to an absence of carcinogens in the small intestine. In order to further examine this hypothesis and to evaluate whether genetic changes that occur in the multistep colon carcinogenesis sequence (8) are also seen in the small bowel, we examined whether the most frequent genetic event in colorectal cancer also is present in small intestinal adenocarcinomas.

In the colon, K-*ras* mutations are an early event in the carcinogenic sequence. K-*ras* mutations are seen frequently in colonic villous adenomas, are present in nearly 50% of colon carcinomas (8), and can even be detected when endoscopic examination of the colon is normal (9). In the colonic mucosa of rodents, carcinogens, such as 1,2-dimethylhydrazine (10), 7,12-dimethylbenzanthracene (11), and NMNU (12) readily cause point mutations in the *ras* genes, and this also can precede morphological changes.

In order to investigate whether small bowel adeno-

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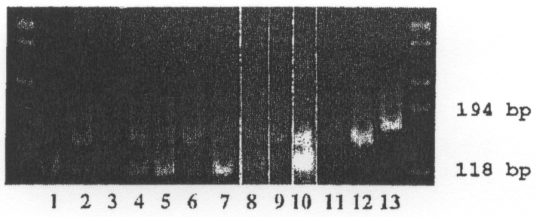

Fig 1. Genomic DNA purified from samples of small bowel adenocarcinomas (lanes 1–3 and 6–10), a colonic polyp, (lane 4), and colonic mucosa (lane 5), and the colon carcinoma cell line SW480 (lanes 12 and 13) was PCR amplified and digested by Bst N1, water control (lane 11), undigested control (lane 13).

carcinomas differ from colon carcinomas in their incidence of K-*ras* codon 12 mutations, we applied a polymerase chain reaction (PCR) -based method to genomic DNA extracted from formalin-fixed paraffin-embedded small bowel tumors that were diagnosed over the past 13 years.

MATERIALS AND METHODS

DNA Preparation. Formalin-fixed, paraffin-embedded sections of small bowel adenocarcinomas were obtained from the Department of Pathology, St. Luke's-Roosevelt Hospital Center. Twelve-micron sections were cut from each block, the paraffin was removed by xylene and ethanol, and the tissue was then incubated in 50 μl lysis buffer (50 mM Tris HCl (pH 8.3), 1 mM EDTA, 0.45% NP-40, and 0.45% Tween 20) containing proteinase K (0.1 mg/ml) for 2 hr at 55°C. This mixture was boiled for 7 min, and 5 ml of the preparation was used as the substrate for DNA amplification. The histopathology of each specimen was reviewed blindly by an independent pathologist. As control, DNA was extracted and amplified for 10 adenocarcinomas of the colon by similar methods.

DNA Amplification. For detection of a mutant codon 12 K-*ras* allele, a single-step PCR amplification was performed using a 5′ end primer mismatching wild-type sequence of codon 12 of the K-*ras* gene, thus creating a Bst N1 restriction enzyme cleavage site (13). Point mutations in either one of the first two nucleotides of codon 12 result in nondigestion by Bst N1. In order to survey Bst N1 digestion, a single nucleotide substitution was also incorporated into the 3′ end primer. The sensitivity of this method was tested by serial dilutions of genomic DNA carrying either wild-type or mutated alleles of K-*ras* codon 12. K-*ras* mutations were still detectable in a 1:16 dilution (13). PCR was carried out as previously described through 40 cycles. PCR products were digested by Bst N1 and electrophoresed through a 8% polyacrylamide gel. Gels were stained with ethidium bromide and photographed on an ultraviolet light transilluminator. A sample containing no genomic DNA was subjected to the same amplification and digestion steps to control possible DNA contamination (Figure 1, lane 11). The colon carcinoma cell line HT29, homozygous for wild-type K-*ras*, was used as control for wild-type sequences. Bst N1 digests wild-type K-*ras* codon 12 sequences at both cleavage sites resulting in a 116-bp band. To distinguish mutated from nondigested bands, we amplified genomic DNA from the SW480 colon carcinoma cell line, which is homozygous for the mutant codon 12 allele either followed by Bst N1 digestion (Figure 1, lane 12) or left undigested (Figure 1, lane 13). Mutated sequences are digested at the 5′ end, but not within codon 12, and give rise to a 143-bp band, whereas complete nondigestion results in a band of 157 bp. The procedure was repeated on two occasions one month apart in amplified specimens and three times in unamplified PCR products with identical results.

RESULTS

The age of the patients examined ranged from 42 to 80 years (mean 61 years); six of the nine patients were male (Table 1). The small bowel adenocarcinomas

TABLE 1. DESCRIPTION OF PATIENTS AND DETAILS OF SMALL BOWEL ADENOCARCINOMAS ANALYZED*

Patient	*Sex*	*Age*	*Specimen*	*Localization*	*Degree of differentiation*	*Stage*	*K*-ras mutation	*Lane in Figure 1*
1	Male	49	Carcinoma (B)	Duodenum		?	–	1
2	Male	50	Carcinoma	Ileum	Well	C	+	2
			Carcinoma	Ileum	Well	C	+	3
			Adenoma	Colon asc.			+	4
			Dist. margin	Colon asc.			+	5
3	Male	54	Carcinoma (B)	Duodenum	Well	B	+	6
			Carcinoma	Duodenum		B	–	7
4	Female	69	Carcinoma (B)	Duodenum	Moderate	C	+	8
			Carcinoma	Duodenum			NA	
			Adenoma	Duodenum			NA	
5	Female	80	Carcinoma (B)	Duodenum		?	+	9
6	Female	69	Carcinoma (B)	Duodenum		?	+	10
7	Male	72	Carcinoma	Duodenum			NA	
8	Male	63	Carcinoma	Ileum			NA	
9	Male	42	Dist. margin	Colon asc.			NA	

*Prevalence of K-*ras* codon 12 mutations detected by mismatch cleavage analysis of PCR amplified K-*ras* alleles, (B), biopsy, NA, not amplified.

were located in the duodenum in six of nine cases, mostly in the periampullary region. From patient 2, who had a large adenocarcinoma of the ileum measuring 6 × 4 cm, we received, in addition to two surgical specimens of the carcinoma, a 0.6-cm colonic polyp and a specimen of colonic mucosa 25 cm distal to the carcinoma. In another patient (patient 3, Table 1) both a biopsy and a surgical specimen from the later resection of the same tumor were available for PCR analysis. Figure 1 shows the bands obtained by two independent PCR amplifications and Bst N1 digestions of mutated and nonmutated K-*ras* codon 12 alleles. DNA amplification was successful in 10 of 17 cases. The successfully amplified samples included some specimens obtained from biopsies that had been stored up to 13 years prior to DNA extraction (Figure 1, lanes 1–10). In contrast to DNA prepared from biopsies, DNA extracted from surgical specimens failed to amplify in some cases (Table 1). This suggests that DNA in these resected specimens might have undergone substantial degradation prior to fixation. As listed in Table 1, a total of six small bowel adenocarcinomas were successfully amplified. Eight of 10 samples displayed mutations of the K-*ras* codon 12 allele (Figure 1, lanes 2–6, 8–10). In one patient (Table 1, patient 2) both an ileal carcinoma and a simultaneously resected small colonic polyp displayed a K-*ras* mutation. Interestingly, in the same patient, microscopically normal-appearing mucosa from the distal resection margin also harbored a K-*ras* codon 12 mutation (Figure 1, lane 5). In one case (Table 1, patient 3) DNA obtained from a biopsy was positive (Figure 1, lane 6) whereas DNA extracted from the surgical specimen of the same tumor was negative for K-*ras* mutations (Figure 1, lane 7), although histological examination confirmed that this specimen contained predominantly carcinoma. A total of five of six small bowel adenocarcinomas were positive for K-*ras* codon 12 mutations. As a positive control, the same method was applied to formalin-fixed, paraffin-embedded specimens of colonic adenocarcinomas in parallel, and K-*ras* mutations were detected in six of 10 carcinomas examined (data not shown).

DISCUSSION

Since small bowel adenocarcinomas are extremely rare, we were able to examine only nine suitable specimens. Tumors in our patients are consistent with those in the literature, demonstrating that they occurred mainly in males, are mostly located in the duodenum, close to the periampullary region, and peak in the seventh decade (4, 14). K-*ras* mutations have been reported in several types of tumors, and their incidence appears to be organ specific (15). In the digestive tract, *ras* mutations are absent in esophageal carcinomas (13, 16), infrequent in gastric cancer (17), but present in over 90% of pancreatic tumors (18). Cholangiocarcinomas display K-*ras* mutations, with a significantly lower frequency than pancreatic cancer (19). In colorectal carcinomas, the incidence of *ras* mutations was found to be nearly 50%, the great majority being K-*ras* codon 12 mutations (13). Our findings suggest that the frequency of K-*ras* codon 12 mutations in small bowel adenocarcinomas is close to that found in colonic or pancreatic cancer. In contrast to our results, a recently published study on *ras* mutations in small bowel tumors failed to reveal K-*ras* mutations in two adenomatous polyps or in one adenocarcinoma (20). The relatively high frequency of K-*ras* mutations present in our series conceivably could be due to DNA contamination or errors of DNA polymerase. However, contamination is unlikely since we obtained negative results in two tumors and did not detect bands using several controls. Furthermore, identical results were reproduced on two separate experiments one month apart. The error rate of Taq polymerase leading to base misincorporations during DNA amplification of $1–2 \times 10^{-4}$ does not explain the relatively high frequency of K-*ras* codon 12 mutations which we have identified (21).

Interestingly, two samples of the same tumor were heterogenous for K-*ras* mutations. Activated K-*ras* is thought to attribute a growth advantage to tumor cells and undergoes positive selection during tumorigenesis. Our finding therefore is surprising and contrasts with a recent study reporting stability of K-*ras* mutations within individual colon carcinomas (22). We have found that K-*ras* mutations not only are frequent in small bowel adenocarcinomas, but also are detectable in histologically normal colonic mucosa, consistent with the observation that K-*ras* mutations can be detected even when colonoscopic examination is normal (9). To explain the low incidence of small bowel tumors, it is likely that mucosal cells might have developed mechanisms protecting themselves against potent carcinogens. Among those mechanisms that may be important are the relative sterility of the bowel, a shortening of the G_1 phase (23), induction of apoptosis by carcinogens (24) and a powerful immune system (25). Learning how epithelial cells of the small intestine circumvent potential cancer-inducing processes could be important in de-

signing protocols for the chemoprevention of colorectal cancer.

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VII. 2

Expression of Cyclins D1 and E in Human Colon Adenocarinomas

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EXPRESSION OF CYCLINS D1 AND E IN HUMAN COLON ADENOCARCINOMAS

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Key Words: Colon carcinomas cell lines, colon tumors, cyclin D1, cyclin E.
Subjects: Patients.
Abbreviations: Cdk = cyclin-dependent protein kinases; poly $(A)^+$ RNA = polyadenylated RNA.

Abstract

Cyclins D1 and E play critical roles in the progression of cells through the G1 phase of the cell cycle. Amplification and/or overexpression of the cyclin D1 gene and aberrant expression of cyclin E have been described in several forms of human cancer. In the present study, we examined the expression of these two genes by Western, Northern and Southern blot analyses in a series of primary human colon carcinomas of various stages and degrees of differentiation and in paired adjacent normal mucosa samples, and also in a series of human colon

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carcinoma cell lines. About 50% of the colon carcinomas displayed a two to five fold increase in the expression of cyclin D1 mRNA and protein, when compared with the paired normal mucosa samples. Six out of eight carcinomas examined showed a four to nine fold increase in cyclin E mRNA and about 50% of the carcinomas displayed a two to three fold increase in cyclin E protein. Low molecular weight cyclin E-related proteins were observed in four out of ten carcinomas. These changes in cyclins D1 and E occurred in both early and late stage tumors. Three of the six cell lines examined displayed a high expression of cyclin D1 mRNA and protein. A very high level of cyclin E mRNA expression was seen in HCT116 cells and this was associated with the presence of low molecular weight cyclin E-related proteins. None of the primary colon carcinomas nor the six cell lines examined displayed amplification of either the cyclin D1 or cyclin E genes. Thus, an aberrant expression of both cyclins D1 and E occurs in a significant fraction of human colon carcinomas.

Introduction

Cyclins were first identified in marine invertebrates as proteins that appear and disappear during the cell cycle (Evans, Rosenthal *et al.*, 1983; Rosenthal, Hunt *et al.*, 1980). Since then at least eight cyclin genes have been identified in mammalian cells. They include: the G1 cyclins, C, D1-3 and E; the S-phase cyclin, A; and the G2/M cyclins, A, B1 and B2. These cyclins act by binding to and stimulating a series of at least six cyclin-dependent protein kinases (cdk). The activities of these cdks are further modulated by protein phosphorylation and dephosphorylation, and by a group of specific cyclin-dependent kinase inhibitors. (For review see Hunter and Pines, 1994; Peters, 1994; Pines, 1994; Sherr, 1993). Cyclins D1 and E were originally cloned by the complemention of G1-functions in yeast mutants deficient in G1 cyclins [Lew, Dulic *et al.*, 1991; Xiong, Connolly *et al.*, 1991). In growth arrested cells, the transcription of both of these cyclins is induced by mitogenic signals. Cyclin D1 is induced in the mid G1 phase (Matsushime, Roussel *et al.*, 1991; Musgrove, Hamilton *et al.*, 1993; Rosenberg, Wong, *et al.*, 1991; Sewing, Buerger *et al.*, 1993; Winston and Pledger, 1993; Won, Xiong *et al.*, 1992) while the induction of cyclin E peaks at the G1/S boundary (Musgrove, Hamilton *et al.*, 1993). The expression of cyclin D1 and cyclin E proteins follows the mRNA

profiles with peaks in the mid and late G1-phase, respectively (Baldin, Likas *et al.*, 1993; Dulic, Lees *et al.*, 1992; Koff, Giordano *et al.*, 1992; Xiong, Zhang *et al.*, 1992). Several studies have demonstrated that cyclins D1 and E are critical for the G1 transition. Cyclin D1 antisense prolongs the G1 phase (Baldin, Likas *et al.*, 1993) and the micro-injection of cyclin D1 antibodies either blocks or inhibits the G1 phase (Baldin, Likas *et al.*, 1993;Quelle, Ashmun *et al.*, 1993; Tam, Theodoras *et al.*, 1994). On the other hand, the overexpression of cyclin D1 (Jiang, Kahn *et al.*, 1993; Ohtsubo, Theodoras *et al.*, 1995) or cyclin E (Jiang, Kahn *et al.*, 1993; Ohtsubo and Roberts, 1993; Ohtsubo, Theodoras *et al.*, 1995) can accelerate the G1 phase.

Cyclin D1 associates with cdks 4 and 6 (Matsushime, Ewen *et al.*, 1992) and cyclin E with cdk2 (Dulic, Lees *et al.*, 1992; Koff, Giordano *et al.*, 1992), thus leading to phosphorylation and inactivation of the Rb tumor suppressor protein, and entry into S-phase (Hinds, Mittnacht *et al.*, 1992; Kato, Matsushime *et al.*, 1993; Meyerson and Harlow, 1994). The Rb protein can induce cyclin D1 transcription, thus providing an auto-regulatory feedback mechanism (Mueller, Lukas *et al.*, 1994). Whereas cyclins D1 and E mediate mitogenic signals, their activities are also negatively regulated through a family of proteins that inhibit the kinase activities of cdk2 and cdk4. These cdkI's are induced by antimitogenic events such as exposure to TGFβ, DNA damage, cell-cell contact, differentiation and senescence (for review see Peter and Herskowitz, 1994).

Both cyclin D1 and E display frequent abnormalities in human tumors. Cyclin D1 is the same gene as PRAD1, a gene rearranged and constitutively activated in parathyroid tumors (Rosenberg, Motokura *et al.*, 1993). Rearrangement of cyclin D1 due to chromosomal translocation has also been seen in lymphomas (Rosenberg, Wong *et al.*, 1991; Williams, Swerdlow *et al.*, 1993). Amplifications and/or an altered expression of the cyclin D1 gene have been reported in a wide range of other types of human tumors, including head and neck squamous carcinomas (Motokura and Arnold, 1993), esophageal cancer (Jiang, Kahn *et al.*, 1992; Jiang, Zhang *et al.*, 1993), liver cancer (Nishida, Fukuda *et al.*, 1994; Zhang, Jiang *et al.*, 1993), breast cancer (Bartkova, Lukas *et al.*, 1994; Gillet, Fantl *et al.*, 1994; Keyomarsi and Pardee, 1993; Keyomarsi, O'Leary *et al.*, 1994; Lebwohl, Muise-Helmericks *et al.*, 1994), non-small cell lung cancer (Schauer,

Siriwardana *et al.*, 1994), and colorectal cancer (Bartkowa, Lukas *et al.*, 1994, 1995; Leach, Elledge *et al.*, 1993). The role of cyclin D1 as a putative oncogene is further supported by the fact that cyclin D1 exerts transforming properties when overexpressed in fibroblasts (Jiang, Kahn *et al.*, 1993), and causes mammary tumors in mice when expressed as a transgene (Wang, Cardiff *et al.*, 1994). There is a close relationship between Rb function and cyclin D1 expression in tumor cells. The cell cycle dependent expression and function of cyclin D1 is preserved in tumors expressing wild-type Rb (Bartkova, Lukas *et al.*, 1994a,b; Jiang, Zhang *et al* 1993; Lukas, Pagano *et al.*, 1994; Lukas, Jadayel *et al.*, 1994). In cells lacking Rb, cyclin D1 levels are low or not detectable, and cyclin D1 function becomes dispensible in these cells (Jiang, Zhang *et al.*, 1993; Lukas, Mueller *et al.*, 1994; Tam, Theodoras *et al.*, 1994). Compared to cyclin D1, there are relatively few studies on cyclin E in human tumors. Although amplification of cyclin E seems to be rare in colon cancer (Leach, Elledge *et al.*, 1993), this gene frequently displays increased and deranged expression in human breast and colon cancers (Gong, Ardelt *et al.*, 1994:Keyomarsi and Pardee, 1993; Keyomarsi, O'Leary *et al.*, 1994).

Interestingly, in colon cancer the Rb gene seems to have a tissue-specific function that contrasts with its general role as a tumor suppressor gene in other tissues. Thus, this gene is expressed in the transitional, proliferating compartment of the colonic crypts and is not detectable in the luminal differentiated cells. Therefore, Rb might have a mitogenic function and/or inhibit differentiation in colonic epithelial cells (Ali, Marcus *et al.*, 1991; Wildrick and Boman, 1994). This hypothesis is supported by the observations that the Rb gene is amplified in about 40% (Gope, Christensen *et al.*, 1990a) and overexpressed in about 70% (Gope, Christensen *et al.*, 1990b) of colon cancers. Since colon cancers do not display point mutations or deletions of the Rb gene, its protein product may play an essential function in colon cancers (Hovig, Smith-Sorensen *et al.*, 1992). The nature of this function and its possible relevance to the known ability of cyclin D1/cdk4 and cyclin E/cdk2 to phosphorylate Rb (3), and thereby regulate its function, are not known.

In view of the above findings, we have examined in detail the expression of cyclins D1 and E at both the mRNA and protein levels, and also the possible amplification of these two genes, in a series of

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primary human colon tumors of various stages and differentiation grades, and also in a series of human colon carcinoma cell lines.

Materials and Methods

Tissue and Cell Culture

Colorectal tumor specimens were received from the Department of Surgery, College of Physicians and Surgeons, Columbia University, and classified as well, moderately and poorly differentiated carcinomas of Dukes' stages A, B and C (Fogh and Trempe, 1975). Specific details are given in the legend to Figures 1 and 2. Normal colonic mucosa was obtained from surgically resected specimens at least 10 cm distal from the tumors, by dissection of macroscopically normal mucosa from the submucosal layer. Tissue samples were immediately frozen in liquid nitrogen and stored at -80^{0}C. The cell lines HT29, HCT116 and SW480 were originally derived from primary human colon carcinomas (Brattain, 1981; Dukes and Bussey, 1958; Fogh and Trempe, 1975). The SW620 cell line was established from a lymph node metastases of an adenocarcinoma; the same primary tumor was the origin of the SW480 cell line (Brattain, 1981). SW480-E8 and SW480-R2 are subclones of SW480 characterized as low and high malignant variants, respectively (Hovig, Smith-Sorensen *et al.*, 1992). Cells were maintained in Dulbecco's modified essential medium containing 5% fetal calf serum.

Southern and Northern Blot Analyses

Genomic DNA was isolated from tissue and cells as described (Leibovitz, Stinson *et al.*, 1976). DNA was digested to completion with either EcoR1 or BamH1. Five µg of digested DNA were electrophoresed on a 1% agarose gel. The samples on the gel were transferred to Hybond-N membranes and hybridization to the indicated probes was performed as described (Jiang, Kahn *et al.*, 1992). For RNA extraction, tissue was homogenized in guanidinium thiocyanate and then immediately centrifuged at 10,000 xg at 4^{0}C for 15 min. For analyzing the cell cultures, the cells were washed with PBS and then scraped into the guanidium thiocyanate solution. The supernatant fractions of the tissue extract and cell lysate were centrifuged through cesium chloride as described (Gross-Bellard, Oudet *et al.*, 1973). Polyadenylated RNA [poly (A)$^+$ RNA] was purified by oligo dT cellulose chromatography (Becton Dickinson, Franklin Lakes, NJ). Five µg of poly (A)$^+$ or 10 µg

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of total RNA was resolved by electrophoresis on a 1% agarose gel containing formaldehyde, and blotted onto Hybond-N membranes (Amersham, Arlington Heights, IL). The membranes were prehybridized with Church buffer and then hybridized with ^{32}P-labeled human cyclin D1 or cyclin E probes, and washed with 2 x SSC, 0.2% SDS for two hours at 65^{0}C. The membranes were exposed to Kodak X-OMAT films for one or up to 14 days. The intensity of the signals was quantitated on a 2-D laser densitometer (Molecular Dynamics, Sunnyvale, CA). The levels of expression in tumors were compared to those in paired normal mucosa, or to the mean values of normal samples, if adjacent normal mucosa was not available. Equivalent loading of the gels for DNA or RNA was confirmed by ethidium bromide staining and all analyses were repeated at least twice and gave similar results.

Western Blot Analysis

Tissue samples and cells cultures were homogenized by sonication in lysis buffer (20 mM Tris-HCL pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 150 mM NaCl, 0.5% Triton X-100) containing 25 mg/mL leupeptin, 25 mg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride, left on ice for 30 min and then centrifuged at 17 000 x g at 4^{0}C. The protein concentrations of the supernatant fraction were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA). One hundred or 300 µg of the tissue extracts and 50 µg of the cell extracts were subjected to 8% polyacrylamide gel electrophoresis. Following electrophoresis, immunoblots were prepared with Immobilon P membranes (Millipore, Bedford, MA) by electrophoretic transfer and blocked in SuperBlock blocking buffer (Pierce, Rockford, IL). Cyclin D1 was detected with a polyclonal (UBI, Lake Placid, NY) or a monoclonal (Santa Cruz Biotechnology, Santa Cruz, CA) anti-cyclin D1 antibody. The expression of cyclin E was detected using a monoclonal antibody raised against cyclin E (Pharmingen, San Diego, CA). The blots were washed, incubated with horseradish peroxidase-conjugated secondary antibodies (1/4000) (Amersham, Arlington Heights, IL) and visualized using the Enhanced Chemi-Luminescence system (Amershan, Arlington Heights, IL) and Kodak X-OMAT films. The intensities of the bands were quantitated on a 2-D laser densitometer (Molecular Dynamics, Sunnyvale, CA). Similar results were obtained in duplicate studies.

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Results

Western Blot Analysis of Cyclins D1 and E in Colon Carcinomas

Protein extracts from samples of carcinomas and paired normal colonic mucosa samples were subjected to Western blot analysis (Figure 1). The colon carcinomas represented Dukes' stages A, B and C and various grades of differentiation. A polyclonal antibody raised against human cyclin D1 detected mainly a doublet of 36 kD and 30 kD cyclin D1 bands. Three out of 11 carcinomas, including a well-differentiated carcinoma of Dukes' A stage, expressed about a three-fold increase in the cyclin D1 protein (Figure 1A, Figure 4A, samples 4, 5, 9), two tumors displayed at least a two-fold increase (samples 2 and 6) and four tumors a 1.5- to two-fold increase (samples 1, 3, 8, 11), when compared to their paired normal mucosa samples. A paired normal sample was not available for tumor sample number seven, but it is apparent that it expresses a moderately high level of cyclin D1 when compared to the other normal samples.

Previous studies indicate that in normal human cells the major cyclin E protein is about 52 kD, and there can also be a less abundant 50 kD protein (Ohtsubo, Theodoras *et al.*, 1995). In the present study, immunoblotting of 10 pairs of colon carcinomas and paired normal colonic mucosa samples, and a metastatic ovarian adenocarcinoma, with a monoclonal anti-human cyclin E antibody detected multiple cyclin E related proteins that ranged in size between 51 kD and 30 kD. The 51 kD protein was the predominant form in most of the tumor and normal mucosa samples (Figure 1B). The level of cyclin E proteins was increased two to three-fold in four of the ten colon tumors (Figure 1B, and Figure 4B, samples 2a, 3a, 4, 5), and these included carcinomas of early stages. In four colon carcinomas, we found an increase in the relative abundance of faster migrating cyclin E related proteins (Figure 1B, samples 1, 2a, 3a, 8). These faster migrating protein bands were faint or absent in the normal mucosa samples. All of the carcinomas that displayed these lower molecular weight bands were moderately differentiated and were Dukes' stage B lesions. Multiple cyclin E-related bands were particularly abundant in the sample of poorly differentiated metastatic ovarian adenocarcinoma that had infiltrated the colon (Figure 1B, sample 11a).

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Northern Blot Analysis for Cyclins D1 and E mRNAs in Colon Tumors

Poly $(A)^+$ RNA was isolated from a tubulo-villous adenoma, from seven colon adenocarcinomas of different stages and differentiation grades, and from paired normal mucosa samples. Northern blot hybridization with a human cyclin D1 cDNA probe revealed mainly a 4.5 kb and a fainter 1.7 kb cyclin band, after overnight autoradiography. Two colon carcinomas, a well-differentiated Dukes' A carcinoma (Figure 2A, Figure 4C, sample 2) and a moderately differentiated Dukes' B carcinoma (sample 3), displayed at least a two-fold increase in cyclin D1 mRNA when compared to the paired normal mucosa samples. A tubulo-villous adenoma (sample 1) and a well-differentiated Dukes' C carcinoma (sample 8) did not display significant increases in cyclin D1 mRNA levels.

To address the question whether colon carcinomas might also display increased levels and/or altered sizes of cyclin E mRNA we hybridized poly $(A)^+$ RNA prepared from eight colon carcinomas and paired normal mucosa samples with a human cyclin E cDNA probe. We detected a single 2.2 kb cyclin E transcript in both the colon carcinoma and normal mucosa samples. The abundance of cyclin E mRNA in both the carcinoma and normal mucosa samples was much lower than that of cyclin D1 mRNA. Thus, autoradiography required one week for detection of cyclin E mRNA, but less than 24 hours for detection of cyclin D1 mRNA.

When compared to the paired normal mucosa samples, five of seven colon carcinomas displayed a three to six-fold, and the sixth sample a nine-fold, increase in the level of cyclin E mRNA (Figure 2B, Figure 4D, samples 2, 3, 5-8). In a tubulo-villous adenoma (sample 1) and in one carcinoma (sample 4) we detected a 1.5-fold increase in cyclin E mRNA. Despite the relatively low levels of expression of cyclin E in the colonic tissues, the relative increase of cyclin E mRNA in these tumors was more pronounced than the relative increase observed with cyclin D1 mRNA. This increase was present in both early and late stage tumors.

Ethidium bromide staining indicated that all lanes were loaded with equal amounts of RNA, and repeated Northern blots gave results similar to those shown in Figure 2.

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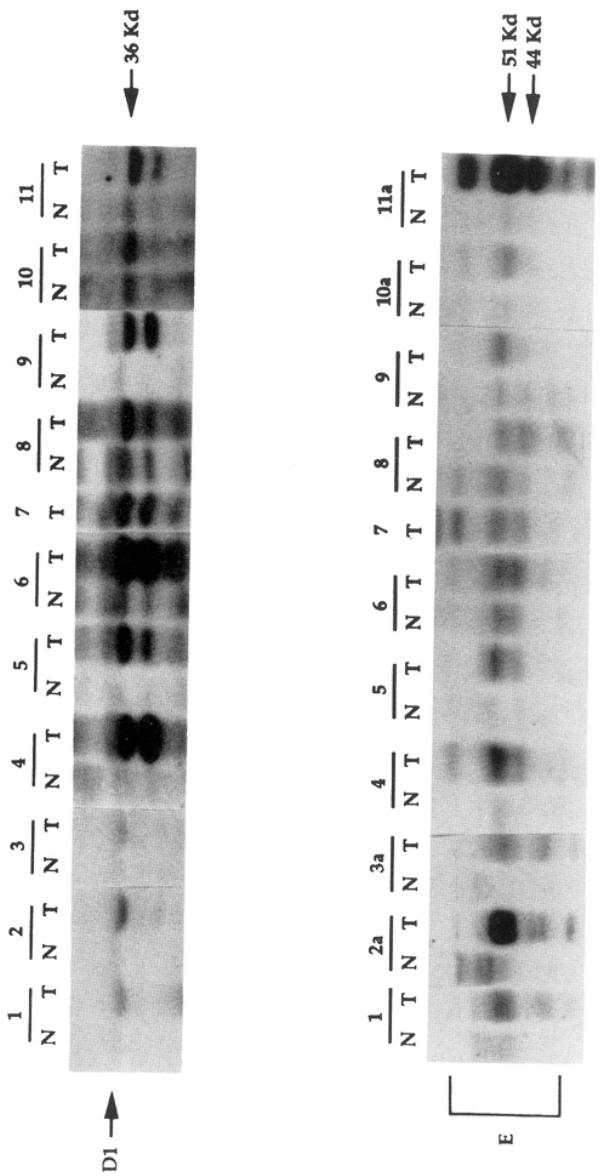

Figure 1: Western blot analyses of cyclin D1 (A) and cyclin E (B) in colon carcinomas (lane T) and paired samples of normal colonic mucosa (lane N). Whole cell lysates were prepared and 300 µg (A) or 100µg (B) of protein were loaded per lane. Cyclin D1 and E proteins were detected using polyclonal anti-cyclin D1 and monoclonal anti-cyclin E antibodies. This analysis included carcinomas of stages Dukes A (samples 1, 2a, 4), Dukes B (samples 5-9), Dukes C (samples 3, 3a, 10, 10a, 11) and a metastasis of an ovarian adenocarcinoma infiltrating the colon (sample 11a). Tumors were further classified as well (samples 3a, 4-6, 10), moderately (samples 1, 2, 2a, 3, 7-9, 10a, 11) and poorly differentiated (sample 11a). Samples 1, 4, 5, 6, 7, 8 and 9 in (A) are the same as the corresponding samples in (B), but this is not the case for the remaining samples. Protein sizes in kilo dalton (kd) are noted at the right.

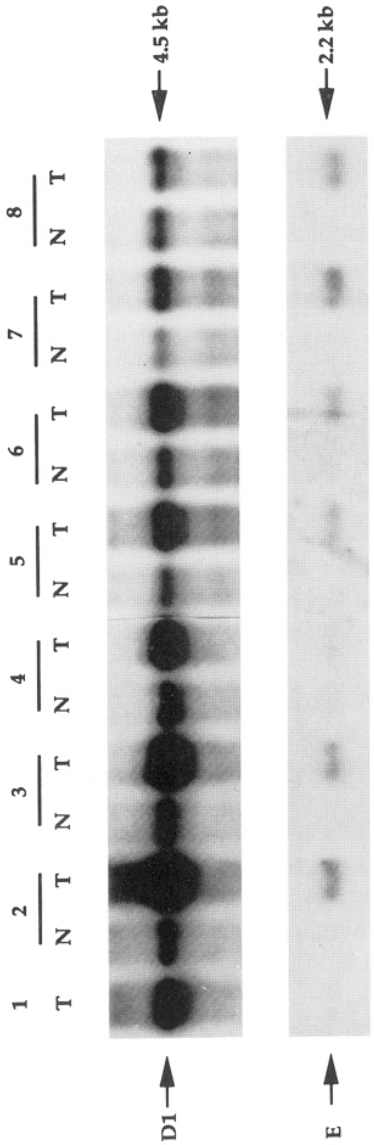

Figure 2: Northern blot analyses of cyclins D1 (A) and E (B) in colon tumors (lane T) and paired normal colonic mucosa (lane N). Five µg of poly $(A)^+$ RNA were loaded per lane. Analyses included a tubulo-villous adenoma (sample 1), colon carcinomas of stages Dukes A (sample 2), Dukes B (samples 3-5) and Dukes C (samples 6-8). Carcinomas were further classified as well (samples 2, 6, 8), moderately (samples 3, 4, 7) and poorly differentiated (sample 5). The samples analyzed for cyclin D1 mRNA are the same as those analyzed for cyclin E mRNA. The tissue samples used in this figure are not the same ones as those analyzed in Figure 1A and 1B, because of the limited amounts of tissue available. Transcript sizes in kilobases (kb) are noted at the right.

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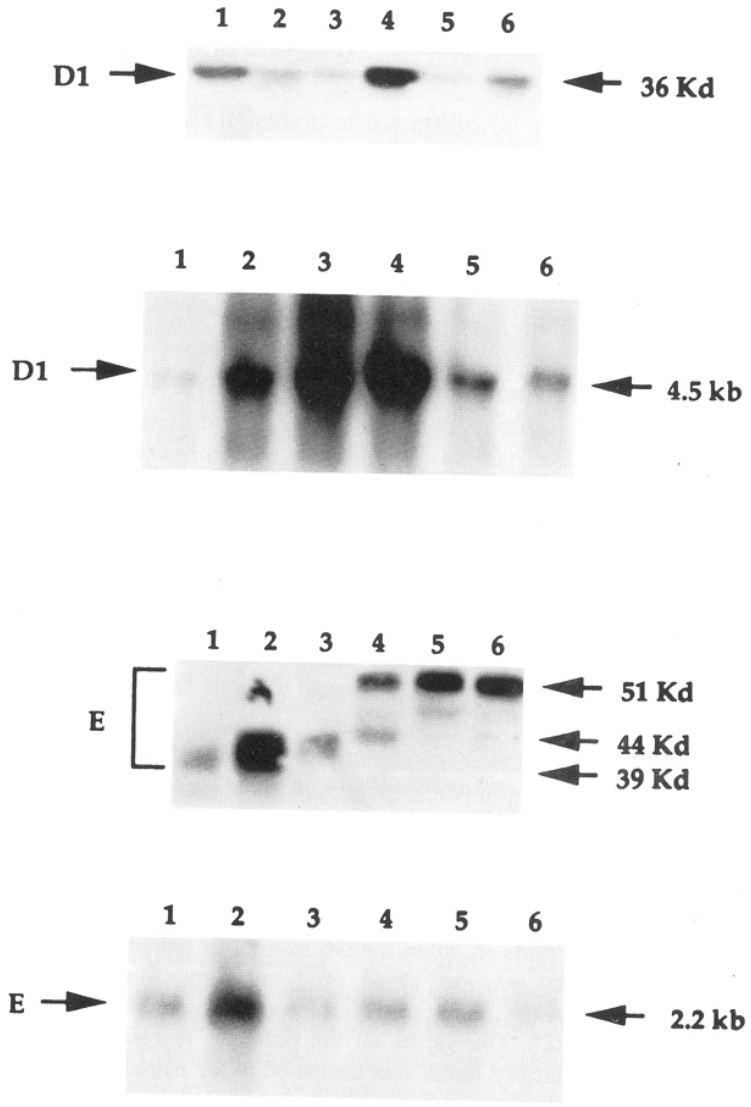

Figure 3: Western and Northern blot analyses of cyclins D1 and E in the colon carcinoma cell lines HT29 (lane 1), HCT116 (lane 2), SW480 (lane 3), SW480-E8 (lane 4), SW480-R2 (lane 5) and SW620 (lane 6). For Western blot analysis 50 µg of protein in a total cell lysate were loaded per lane and cyclin D1 (A) and E (C) proteins were detected using monoclonal anti-cyclin D1 (Santa Cruz) and anti-cyclin E (Pharmingen) antibodies, respectively. Protein sizes in kilo dalton (kd) are noted at the right. For Northern blot analysis 10µg of total RNA was loaded per lane and hybridized to cDNA probes specific to cyclin D1 (B) and cyclin E (D). Transcript sizes in kilobases (kb) are noted at the right.

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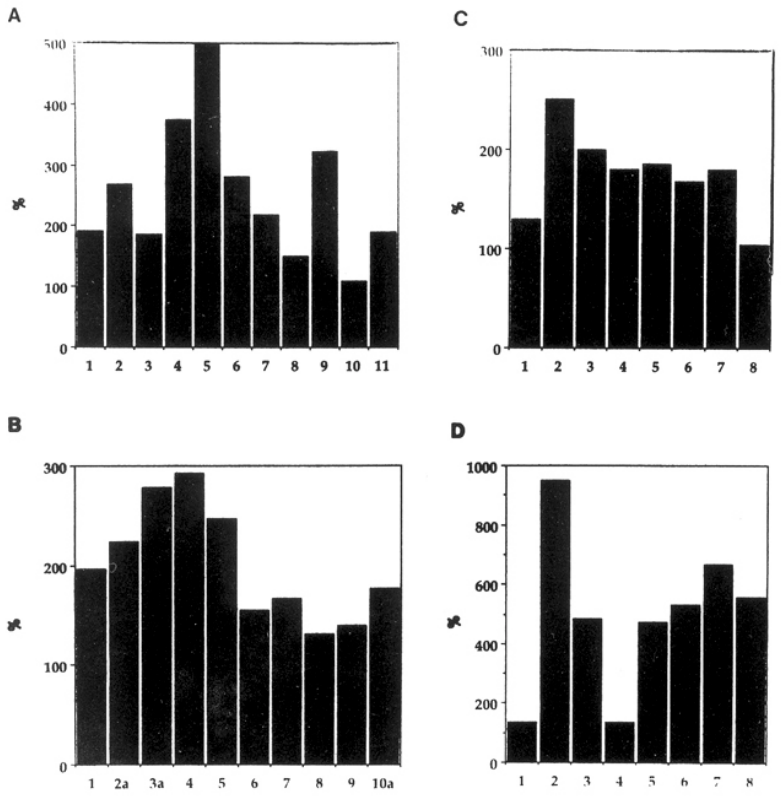

Figure 4 (A-D): Quantification by 2-D laser densitometry of cyclin D1 and E mRNA and protein levels in colon tumors relative to paired normal colonic mucosa. Densitometry was performed on x-ray films from Western and Northern blot experiments shown in Figures 1A (A), Figure 1B (B), Figure 2A (C) and Figure 2B (D). The values on the ordinates represent the per cent increase when the indicated tumor was compared to the paired normal sample. Note the different scales in each figure. Characteristics of the individual tumors are described in the legends to Figures 1 and 2. Sample 11a from Figure 1B is not included since it was a metastatic ovarian cancer rather than a primary colon tumor.

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Western Blot Analysis of Cyclins D1 and E in Human Colon Carcinoma Cell Lines

The levels of expression of the cyclin D1 protein in a series of six human colon carcinoma cell lines varied markedly among the cell lines studied. The highest level was found in SW480-E8 cells (Figure 3A, lane 4), which was greater than ten-times the level of the more malignant subclone SW480-R2 (lane 5). Three of the six cell lines expressed cyclin E proteins that were mainly in the 39 to 44 kD size range, and lacked the 51 kD protein (Figure 3C, lanes 1-3). The SW480 cells (lane 3) expressed the faster migrating protein forms, but its subclones SW480-E8 (lane 4) and SW480-R2 (lane 5) expressed mainly the 51 kD protein. None of the cell lines expressed the less than 39 kD protein bands detected in four out of the ten primary colon carcinomas.

Northern Blot Analysis of Cyclin D1 and E mRNAs in Colon Carcinoma Cell Lines

Cyclin D1 mRNA was abundantly expressed in all of the cell lines examined. The 4.5 kb cyclin D1 transcript was readily visible by loading 10 µg of total RNA and overnight autoradiography of the Northern blot (Figure 3B). Nevertheless, there were considerable differences in the levels of this mRNA among the cell lines and these levels did not always correlate with the levels of cyclin D1 protein. SW480-E8 cells expressed relatively high levels of both the cyclin D1 mRNA and cyclin D1 protein when compared to SW480-R2 cells (compare lanes 4 and 5 in Figure 3A and 3B). The doubling times of SW480-E8 and SW480-R2 cells were 32 hours and 15 hours, respectively (Tomita, Jiang *et al.*, 1992). Thus, the level of cyclin D1 expression was inversely related to the growth rates of these two subclones. Ethidium bromide staining indicated that lanes 2-6 in Figure 3B and 3D were loaded with equal amounts of RNA, but the relatively faint band in lane 1 is due to underloading of this sample.

Like the primary colon carcinomas, all of the colon carcinoma cell lines expressed a single cyclin E mRNA transcript of 2.2 kb (Figure 3D). The abundance of cyclin E mRNA was low compared to that of cyclin D1 since one-week of autoradiography was required for detection of the cyclin E mRNA. We found considerable variations in the amount of cyclin E mRNA among the six cell lines studied. The HCT 116 cell line that was derived from a patient with hereditary non-polyposis colon

cancer displayed a very high level of cyclin E mRNA expression (Figure 3d, lane 2).

Southern Blot Analysis of Cyclins D1 and E

To investigate whether amplification of either the cyclin D1 or E genes might contribute to the increased expression of both of these genes in the colon tumor cells, high-molecular-weight DNA was prepared from 18 colon adenocarcinomas, 15 samples of adjacent normal colonic mucosa, and the six colon cancer cell lines. For analysis of the cyclin D1 gene, DNA was digested with EcoR1. This resulted in readily identifiable restriction fragments of 4.0 kb, 2.2 kb and 2.0 kb. Analysis of the cyclin E gene was based on the detection of a 5 kb and 15 kb fragment obtained after BamH1 digestion. None of these samples displayed detectable amplification of either the cyclin D1 or cyclin E genes (data not shown). Previous studies also failed to detect amplification of the cyclin D1 gene in a series of colon cancer cell lines (Leach, Elledge *et al.*, 1993).

Discussion

The normal colonic epithelium is one of the tissues that has the fastest turnover rates. Continuous renewal of the colonic mucosal cells is maintained through a stem cell compartment, located at the base of the colonic crypts. Cells in the upper region of the crypt undergo differentiation and apoptosis. Colon tumor cells have apparently escaped this orderly pattern although under certain conditions they can display differentiation (for review see Choi, Tchou-Wong *et al.*, 1990; Velcich, Palumbo *et al.*, 1995). Because of the critical role of cell cycle control in cell proliferation and differentiation, in the present study we examined the expression of two important G1 cyclins, cyclins D1 and E, in a series of primary human colon carcinomas and colon carcinoma derived cell lines. We detected increased levels of cyclin D1 expression at both the mRNA and protein levels in more than 50% of the primary colon carcinomas and relatively high levels in the six cell lines examined. These findings are consistent with previously published data based on the immunostaining of paraffin embedded sections of primary colon carcinomas (Bartkova, Lukas *et al.*, 1994a) and Western blot analysis (Bartkova, Lukas *et al.*, 1995), with an anti-human cyclin D1 antibody, but neither of the previous studies included Northern or Southern blot

analyses of cyclin D1 on primary colon carcinomas. In recent studies, we have also confirmed the present findings of increased expression of the cyclin D1 protein in tumor cells, by immunohistochemistry, and demonstrated that the increased expression is confined to the carcinoma cells and not due to contaminating normal mucosa or stromal cells (Arber, Hibshoosh *et al.*, 1996). The increased expression of cyclin D1 in adenocarcinomas of the colon is not simply due to increased cell proliferation since in this and previous studies the increased expression does not correlate with tumor stage or degree of differentiation. In addition in our study employing immunostaining (Arber, Hibshoosh *et al.*, 1996), there was no correlation between the extent of cyclin D1 expression and the mitotic index of the tumors. Furthermore, the levels of cyclin D1 expression in the series of human colon carcinoma cell lines examined in the present study (Figure 3) did not correlate with their doubling times (data not shown). In this sense, the increased expression of cyclin D1 in a subset of human colon carcinomas is aberrant.

Since in the present study we did not detect amplification of the cyclin D1 gene in colon tumors, in contrast to the situation frequently seen in human cancers of the esophagus and breast (see Introduction), the increased expression of cyclin D1 in colon tumors appears to be due to increased *de novo* transcription or increased mRNA stability. Further studies are required to distinguish these two alternatives. Because of the limited amount of tissue, it was not possible to do protein and RNA analyses on the same sets of normal mucosa and colon tumor samples. However, our parallel studies on the six colon tumor cell lines indicated that the extent of increase in cyclin D1 mRNA did not always correlate with the extent of increase of cyclin D1 protein. Therefore, regulation of expression at the translational and post-translational levels may also play a role. Since certain growth factors, the tumor promoter TPA, and estrogen can induce cyclin D1 expression in various types of cells (Musgrove, Hamilton *et al.*, 1993; Sewing, Buerger *et al.*, 1993; Winston and Pledger, 1993; Won, Xiong *et al.*, 1992), the increased expression of cyclin D1 seen in colon tumor cells could reflect the constitutive activation of growth factor related signal transduction pathway(s). An alternate possibility is related to recent evidence that the Rb protein can induce cyclin D1 expression (Mueller, Lukas *et al.*, 1994), since as discussed in the Introduction, the Rb gene is often

amplified and overexpressed in human colon carcinomas (Gope, Christensen *et al.*, 1990a,b), and Rb can induce an increased expression of cyclin D1 (Mueller, Lukas *et al.*, 1994). Alternatively, the increased expression of cyclin D1 in colon tumors might be a consequence of activation of the c-K-*ras* oncogene, which frequently occurs in human colon cancers (Vogelstein, Fearon *et al.*, 1988). This possibility is consistent with recent studies indicating that transformation of the rat enterocyte cell line ICE-18 by either activated c-H-*ras* (Filmus, Robles *et al.*, 1994) or c-K-*ras* (Arber, Sutter *et al.*, 1996) oncogenes results in increased expression of cyclin D1. In view of these findings, it will be of interest in future studies to determine whether the subset of colon carcinomas that display an increased expression of cyclin D1 also displays an increased expression of the Rb protein and/or altered levels of phosphorylation of the Rb protein, and/or activating mutations in the c-K-*ras* oncogene.

Regardless of the underlying mechanism, the increased expression of cyclin D1 in colon carcinomas could contribute to their abnormal growth, since in model cell culture systems overexpression of cyclin D itself (Jiang, Kahn *et al.*, 1993) or together with an activated *ras* oncogene (Hinds, Dowdy *et al.*, 1994) enhances malignant cell transformation. Furthermore, stable expression of an antisense cyclin D1 sequence can revert the transformed phenotype of a human esophageal carcinoma cell line in which the cellular cyclin D1 gene is amplified and otherwise overexpressed (Zhou, Jiang *et al.*, 1995). The precise mechanisms underlying these effects on transformation are, however, not known at the present time. Increased expression of cyclin D1 in colon tumors might render cells more resistant to the growth inhibitory effects of TGFβ on normal epithelial cells, since this inhibitory effect appears to be mediated, at least in part, through inhibition of cyclin D1/cdk4 kinase activity (Peters, 1994). Indeed, overexpression of an exogenous cyclin D1 in an immortalized human esophageal cell line increases the resistance of these cells to inhibition by TGFβ (Okamoto, Jiang *et al.*, 1994).

We should emphasize that the levels of overexpression of cyclin D1 in colon cancer are at most a few fold and that this is also usually the case in esophageal cancer cells in which the cyclin D1 gene is also amplified (Jiang, Zhang *et al.*, 1993). These findings suggest that high levels of expression of cyclin D1 might inhibit cell growth. Indeed, this

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has been seen in some cell systems (Han, Sgambato *et al.*, 1995; Quelle, Ashmun *et al.*, 1993. This inhibitory effect could be due to the binding of cyclin D1 to PCNA or other proteins involved in DNA synthesis (Han, Sgambato *et al.*, 1995; Pagano, Theodoras *et al.*, 1994). These findings might explain why we saw increased but not very high levels of expression of cyclin D1 in colon tumors.

In addition to the finding of increased levels of cyclin D1, in the present study we also observed an increased expression of cyclin E, at both the mRNA and protein levels in colon tumors, when compared to paired samples of normal colonic mucosa (Figures 1, 2 and 4). Previous investigators have also observed increased levels of the cyclin E protein in a variety of human tumors, as well as deregulation of its time of appearance during the cell cycle (Gong, Ardelt *et al.*, 1994; Keyomarsi, O'Leary *et al.*, 1994). Whereas the predominant cyclin E protein usually expressed in normal human cells is 52 kD (Ohtsubo, Theodoras *et al.*, 1995), in the present study we found that colon tumors frequently expressed lower molecular weight forms of this protein which ranged in size from 44 to 39 kD (Figure 1B). These lower molecular weight cyclin E proteins were also seen in some human colon carcinoma cell lines (Figure 3C). Keyomarsi and Pardee (1993) have also described the presence of these lower molecular weight forms of cyclin E in various human tumors, particularly in aggressive and highly metastatic tumors of the stomach and ovary and in breast cancers associated with a poor prognosis (Keyomarsi, O'Leary *et al.*, 1994). It is highly unlikely that these additional protein bands are simply cross-reacting proteins since they are detected with different cyclin E antibodies by both our group and other investigators (Keyomarsi, O'Leary *et al.*, 1994), and are specific for certain tumors. The origin of these lower molecular weight forms of cyclin E is not known. It is of interest that synchronized HeLa cells express cyclin E proteins which range in size between 51 and 55 kD as they progress through the cell cycle (Kulic, Lees *et al.*, 1992). In addition, the expression of a full-length exogenous cyclin E cDNA in rat fibroblasts results in the synthesis of 39 kD and 44 kD proteins, whereas in the same cells the endogenous cyclin E is expressed exclusively as a 51 kD protein (Ohtsubo, Theodoras *et al.*, 1995). Splicing of the endogenous cyclin E mRNA has been described in *Drosophila* (Sewing, Roenicke *et al.*, 1994), and in human cells (Dukes and Bussey, 1958). In the latter case, this leads to the formation of 43 and 48 kD proteins, and

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not the lower molecular weight forms seen by us and Keyomarsi and Pardee (1993). Furthermore, in both the normal colonic mucosa and colon tumor samples we detected only the characteristic 2.2 kb cyclin E mRNA (Figure 2B). These lower molecular weight forms might be due to post-translational processing, but non-specific proteolytic degradation also remains as a possible explanation. Thus, their precise origin and functional significance remain to be determined.

Thus, the present studies, taken together with previous studies, indicate that an increased expression of cyclins D1 and E, and the occurrence of low molecular weight forms of the cyclin E protein, frequently occur in human colon carcinomas. In recent studies, we have found that an increased expression of cyclin D1 can be detected by immunohistochemistry in about 34% of adenomatous polyps of the colon (Arber, Hibshoosh *et al.*, 1996). Further studies are required to determine whether these changes play a role in the process of tumor progression, are significant with respect to prognosis, or provide novel targets for therapy.

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VII. 3

Increased Expression of Cyclin D1 and the RB Tumor Suppressor Gene in c-K-ras Transformed Rat Enterocytes

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Increased expression of cyclin D1 and the RB tumor suppressor gene in c-K-*ras* transformed rat enterocytes

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Activating mutations in the c-K-*ras* gene occur in about 40% of human colorectal carcinomas, yet the role of this oncogene in tumorigenesis is not known. We have developed a model cell culture system to study this problem, utilizing the immortalized but non-tumorigenic epithelial cell line IEC18, originally derived from normal rat intestine epithelium. These cells were cotransfected with the drug resistance selectable marker tk-*neo* and the plasmid pMIKcys, which encodes a mini human c-K-*ras* gene (15 kb) containing a cysteine mutation at codon 12. Drug resistant clones were isolated. Clones which also expressed the activated c-K-*ras* gene displayed a transformed morphology, decreased doubling time, increased level of diacylglycerol, anchorage independent growth in soft agar and an aneuploid karyotype and they were also tumorigenic when injected into nude mice. These clones also displayed increased expression, at both the mRNA and protein levels, of cyclin D1 and Rb. These findings may be of clinical relevance since human colorectal tumors also frequently display increased expression of both cyclin D1 and Rb. This model system may be useful for understanding the role and interrelationship between activation of the c-K-*ras* oncogene and increased expression of cyclin D1 and Rb in colorectal tumorigenesis.

Keywords: retinoblastoma; cyclin D1; *ras*; colon cancer

Introduction

Activating mutations at codons 12 or 13 of the c-K-*ras* proto-oncogene occur in about 40% of human colorectal cancers. They are frequently first detected in adenomatous polyps and occur with about equal frequency in Dukes A, B, C and D carcinomas. Thus, they often represent a fairly early and persistent event in the multistage process of colorectal carcinogenesis (for review see Fearon, 1993; Fearon and Vogelstein, 1990). However, very little is known about the functional effects of the activated c-K-*ras* oncogene with respect to the growth and progression of colorectal tumors. Recent studies indicate that a major role of the p21 ras protein is to couple the effects of certain growth factors to the raf – map kinase kinase – map kinase signal transduction pathway, thus leading to nuclear expression of several early response genes. The p21 ras proteins can also play a role in alternative signal transduction pathways (for review see Bokoch and Der, 1993; Marshall, 1993; Chambers and Tuck, 1993; Prendergast *et al.*, 1995). These specific aspects have not been examined in colon tumors. At the same time, there is increasing evidence that many types of tumors display abnormalities not only in agonist-induced pathways of signal transduction, but also in events that directly regulate the G1/S transition in the cell cycle. The Rb tumor suppressor gene plays a critical role in regulating this transition. The Rb protein blocks the G1/S transition, but phosphorylation of this protein by cyclin D1/CDK4 and cyclin E/CDK2 inactivates this inhibitory function thus permitting cell cycle progression (Hinds *et al.*, 1992; Kato *et al.*, 1993). A variety of human tumors frequently display inactivating mutations in the Rb gene, thus abrogating this type of check point control. A curious exception is the fact that only infrequently (in about 10% of cases) do human colon carcinomas show inactivation or mutations of the Rb gene (Sherr, 1993; Hoving *et al.*, 1992). In fact, there is the paradoxical finding that human colon carcinomas often display increased expression (2–3-fold) of the Rb gene. In some cases this appears to be due to an increased number of copies of chromosome 13 and in others there is actual amplification of the Rb locus (Sherr, 1993; Lothe *et al.*, 1992; Gope *et al.*, 1990; Wildrick and Boman, 1994). It is of interest that cyclin D1, which plays a role in the phosphorylation of the Rb protein, is often overexpressed in a variety of human tumors, either because of rearrangements or amplification of the corresponding gene (Keyomarsi and Pardee, 1993; Pines and Hunter, 1994; Jiang *et al.*, 1993; Zhang *et al.*, 1993). This appears to provide an alternate mechanism for over-riding the inhibitory effects of Rb (Jiang *et al.*, 1993). Increased expression of cyclin D1, at both the mRNA and protein levels, is also frequently seen in human colon carcinomas, although in these tumors the gene is usually not rearranged or amplified (Bartkova *et al.*, 1994; Arber *et al.*, 1996; Sutter *et al.* unpublished studies). It seems likely, therefore, that this reflects increased transcription of the cyclin D1 gene, perhaps as a result of mutations in another gene(s).

In view of the above findings we thought that it would be of interest to determine whether an activated c-K-*ras* gene might influence the expression of both cyclin D1 and Rb in cultured intestinal epithelial cells. Since normal human or rodent colonic epithelial cell lines are not available, we have used as a model system

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the IEC-18 enterocyte cell line originally isolated from normal rat ileum. The present study indicates that derivatives of this cell line transformed by an activated human c-K-*ras* oncogene display increased expression of both cyclin D1 and Rb genes, thus revealing novel effects of this oncogene which may be relevant to colorectal carcinogenesis.

Results and Discussion

Although it is an immortalized cell line, the parental IEC-18 cells demonstrate a normal epithelial phenotype. In contrast, the three clonal derivatives, IEC-18-R1, -R4 and R-10 that stably express the activated c-K-*ras* oncogene showed properties associated with malignant cell transformation (Table 1). Thus, all three of these clones had a refractile and fusiform morphology, in contrast to the flat and epithelioid morphology of the parental cells. The parental cells did not form colonies when grown in 0.3% agar suspension, thus demonstrating complete anchorage-dependence, but all three of the *ras*-transformed clones did form colonies in soft agar, although they did so with a low and variable cloning efficiency. The transformed but not the parental cells were tumorigenic when injected into nude mice. When grown as monolayer cultures the IEC18-R1 and -R4 clones displayed a decreased exponential doubling time, but the IEC18-R10 clone showed an increased doubling time. The properties of the c-K-*ras* transformed IEC18-R1 and -R4 clones are consistent with previous studies indicating that IEC18 cells can be transformed by an activated c-H-*ras* gene (Buick *et al*., 1987). The latter cells also had a fusiform morphology, a shorter doubling time and displayed anchorage independent growth. Furthermore; they expressed TGFα and yet they continued to express intestinal-specific antigenic markers (Buick *et al*., 1987; Zhao and Buick, 1993).

Because there is evidence that protein kinase C (PKC) mediates the action of several growth factors and also interacts with the *ras*-signalling pathway (Price *et al*., 1989; Nishizuka, 1993) we also studied the effects of 10 mM TPA on the growth of these cells in monolayer culture. The addition of this concentration of TPA to the parental IEC 18 cells had no appreciable effect on growth. However, TPA markedly inhibited the growth of the IEC18-R1 and -R4 clones, but not the -R10 clone. Previous studies indicate that *ras*-transformed fibroblasts can display increased cellular levels of diacylglycerol (DAG), an activator of PKC, presumably through activation of a cellular phospholipase (Pai *et al*., 1991; Price *et al*., 1989). Therefore, we also examained this parameter. The IEC18-R1 and -R4 clones showed increased levels of DAG, when compared to the IEC18 cells, but the IEC18-R10 cells demonstrated an appreciable decrease in DAG (Table 1). Thus, the IEC18-R10 clone is unusual since in contrast to the other two *ras*-transformed clones it has an increased doubling time and is not inhibited by TPA when grown in monolayer culture and has a decreased level of DAG, even though it shares with the other two clones other properties of transformed cells (Table 1).

Since, as mentioned in the Introduction, the expression of cyclin D1 is frequently increased in a variety of human tumors, including colon carcinomas, we next examined the expression of this gene in this series of cell lines. Northern blot analyses indicated that the IEC18 parental cells expressed only trace amounts of the 4.7 kb cyclin D1 mRNA, whereas all three of the *ras*-transformed clones expressed abundant levels of this mRNA (Figure 1A). Western blot analysis indicated that all three of the *ras*-transformed clones also expressed a marked increase in the amount of the 36 kD cyclin D1 protein (Figure 1A). Southern blot analyses indicated that none of the three *ras*-

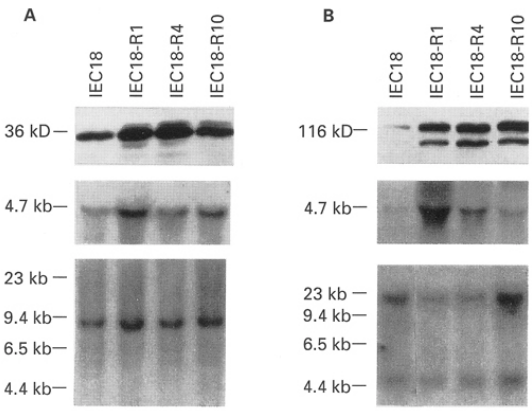

Figure 1 Increased expression of the endogenous cyclin D1 and Rb genes in c-K-*ras* transformed IEC18 cells. Shown are Western (top), Northern (middle) and Southern (bottom) blots for the detection of (**A**) cyclin D1, or (**B**) Rb protein, mRNA, and DNA levels in the parental IEC18 and c-K-*ras* transformed IEC18-R1, -R4 and -R10 cell lines. Cell lines are indicated at the top and molecular sizes are indicated on the left. For further details, see Materials and methods. Blots were first hybridized to the cyclin D1 probe washed, and then hybridized to the Rb probe

Table 1 Morphological and functional properties of the parental and *ras* transformed IEC-18 cell lines

Cell line	*Morphology*	*Growth in soft Agar* (%)*	*Doubling time* (h)	*Intracellular Diacylglycerol*†	*Inhibition by TPA*‡	*Tumorigenicity in nude mice*
IEC18	Flat and epithelioid	0	20.7	2.93	0	0/4
IEC18-R1	Refractile and fusiform	1.06	10.2	9.09	4+	4/4
IEC18-R4	Flat/refractile and fusiform	0.03	18.0	4.74	4+	–
IEC18-R10	Refractile and fusiform	0.53	25.2	0.93	0	4/4

*Expressed as cloning efficiency. † nmol of DAG/10^7 cells. ‡ 10 nM TPA

transformed clones displayed amplification of the cyclin D1 gene (Figure 1A). Thus, the increased expression of cyclin D1 seen in these cells resembles the situation in primary human colon carcinomas in which cyclin D1 is often overexpressed, in the absence of amplification of this gene (Leach *et al.*, 1993; Bartkova *et al.*, 1994; Arber *et al.*, 1996). During the course of these studies Filmus *et al.* (1994) reported that IEC18 cells transformed by an activated c-H-*ras* oncogene also display increased expression of cyclin D1 in the absence of gene amplification. Thus, both activated c-K-*ras* and c-H-*ras* genes can produce this effect in these cells.

Because of the usual reciprocal roles of cyclin D1 and Rb in controlling the G1/S transition (see Introduction), we thought it would be of interest to extend the findings on cyclin D1 made by us, and by Filmus *et al.* (1994), by also analysing the expression of the Rb gene, by both Northern and Western blot analysis. We were surprised to find that all of the three *ras*-transformed IEC18 clones demonstrated a marked increase in the levels of Rb mRNA and protein (Figure 1B). We assume that the more slowly migrating band seen in the doublet of proteins that reacts with the anti-Rb antibody used in these studies represents the hyperphosphorylated Rb protein. However, this remains to be established since to our knowledge the Rb proteins expressed in these rat cells have not been previously characterized. Utilizing a probe specific for the Rb sequence we were unable to detect by Southern blot analysis any evidence of amplification of the Rb locus in the c-K-*ras*-transformed cells (Figure 1B). We should emphasize that G418 resistant IEC-18 cells obtained after transfection with only the *neo* gene (without the c-K-*ras* gene) did not display a transformed phenotype or increased expression of the cyclin D1 or Rb mRNA or proteins when compared to the parental cells (data not shown). Although the IEC18-R1 cells expressed the highest levels of Rb mRNA these cells did not express the highest levels of the Rb protein. Thus, the expression of the Rb protein in these cells appears to be under complex control at several levels.

There is evidence that increased expression of cyclin D1 in human tumors always occurs in tumors that continue to express the Rb protein (Jiang *et al.*, 1993). Furthermore, there is evidence that increased expression of Rb can induce the expression of cyclin D1 (Muller *et al.*, 1994), presumably because the cyclin D1 promoter contains binding sites that can be modulated by the Rb protein (Muller *et al.*, 1994). Based on these studies we postulate the existence of three possible pathways downstream of c-K-*ras*: (a) c-K-*ras* induces the expression of Rb and cyclin D1 independently; (b) c-K-*ras* induces the expression of cyclin D1, which increases the expression of Rb; or (c) c-K-*ras* induces the expression of Rb, which increases expression of cyclin D1. In order to distinguish between these possibilities, we developed derivatives of the IEC18 cell line that stably express high levels of cyclin D1 mRNA and protein. However, these cells did not express increased levels of the Rb protein, did not show a transformed phenotype, and were not tumorigenic when injected into nude mice (data not shown). These results provide evidence against pathway (b). Studies are in progress to examine pathways (a) and (c).

To extend the above analysis we also performed cytogenetic studies on the control IEC18 parental cells and the transformed IEC18-R1 and IEC18-R10 clones. Karyotype analysis of trypsin-giemsa banded metaphase figures of the control IEC18 cells (Figure 2A) indicated that they had a modal number of 45 chromosomes. In addition to the near diploid karyotype of the rat (which contains 42 chromosomes), there was an extra copy of chromosomes 3 and 20, and an unidentifiable medium-sized acrocentric marker chromosome. No rearrangements were identified apart from the marker chromosome. The IEC18-R1 cell line had chromosome numbers varying from 77 to 89 (Figure 2B). Most of the chromosomes were present in 3–4 copies. No major rearrangements were identified except for one to two copies of an acrocentric marker chromosome. Three normal appearing copies of chromosome 15 (which contains the Rb locus) were present in most of the cells. The IEC18-R10 cell line had a chromosome number that varied between 63 to 67 (Figure 2C). Most of the chromosomes were present in three copies. A variety of rearranged chromosomes were seen in some cells but not others, including a long apparently dicentric chromosome derived from chromosome 1, an apparent inversion of chromosome 4 and two metacentric Robertsonian translocations (10/8), were present in some cells but not others. Chromosome 15 occurred in 3–4 copies. Thus, the karyotype data show that c-K-*ras*-transformation of the IEC18 cell line, which has almost a normal diploid rat chromosome complement, was accompanied by the development of a high degree of aneuploidy, as well as several chromosome rearrangements. Rearrangements were more numerous in the IEC-R10 cell line than in the IEC18-R1 cell line, and the latter cell line also showed a higher degree of karyotypic heterogeneity between individual cells. The large number of chromosome rearrangements in the IEC-R10 cell line may explain its slower growth rate. There were no obvious homogeneously staining regions (HSR) in either of the *ras*-transformed cells.

Thus, the present studies indicate that transformation of the IEC-18 cells with an activated c-K-*ras* oncogene leads to highly pleiotropic changes including aneuploidy and numerous chromosomal rearrangements. Our findings may be relevant to human colorectal cancer since, as mentioned in the Introduction, increased expression of the Rb gene is seen in a major fraction of primary human colon carcinomas (Gope *et al.*, 1990; Lothe *et al.*, 1992; Wildrick and Boman, 1994). This seems paradoxical since the Rb gene is usually considered a tumor suppressor gene which exerts negative control on cell cycle progression. On the other hand it is possible that in colonic epithelial cells, and certain other cell types, this inhibitory effect is required to prevent transformed cells from undergoing apoptosis, due to other imbalances in the circuitry that regulates their cell cycle. An alternative possibility is that genes near the Rb locus which are co-amplified and/or co-expressed with Rb in some colorectal cancers (Lothe *et al.*, 1992) might exert a positive effect on growth. The present model system may be useful for elucidating the functional significance of increased expression of both cyclin D1 and the Rb tumor supressor gene in human colon carcinogenesis.

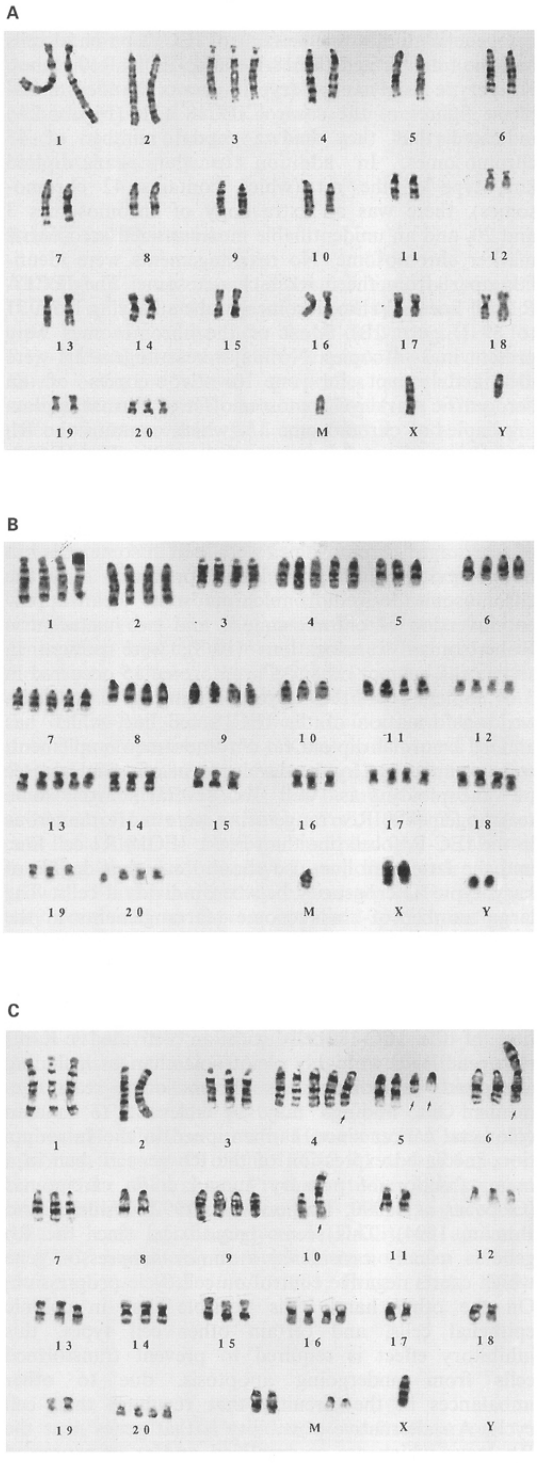

Figure 2 Karyotypes of (**A**) IEC18 cells (**B**) IEC18-R1 cells and (**C**) IEC18-R10 cells, showing that the transformation of the IEC18 cell line, which has close to a normal diploid rat chromosome complement, was accompanied by the development of a high degree of aneuploidy and chromosome rearrangements. 'M' indicates marker chromosomes of unidentified origin. Arrows indicate rearranged but identifiable chromosomes

Materials and methods

Cell culture procedures

To generate the c-K-*ras* transformed IEC18 clonal cell lines, pMIKcys, a plasmid encoding a mini human c-K-*ras* gene (Kahn *et al*., 1987) and pMV7, a retroviral vector encoding a neomycin resistance gene under the transcriptional control of the herpes simplex virus thymidine kinase promoter, were co-transfected at a 5:1 ratio into exponentially growing IEC18 cells, with the lipofectin reagent (Gibco-BRL, Grand Island, NY) under conditions described by the supplier. pMIKcys encodes 15 kb of human c-K-*ras* sequences derived from the PR371 lung carcinoma cell line, including the promoter region and exons Φ, 1,2,3 and 4B but has major deletions within intron sequences. pMIKcys also possesses an activating cysteine mutation at codon 12 (Kahn *et al*., 1987). Neomycin resistant colonies were isolated from a single plate following selection in Dulbecco's Minimal Essential Medium (DMEM) and 5% fetal calf serum (FCS) plus 800 μg/ ml G418 (Gibco-BRL, Grand Island, NY), and maintained in DMEM plus 200 μg/ml G418, unless noted. Three of these clonal cell lines, IEC18-R1, -R4, and -R10, which were shown to express pMIKcys directed human c-K-*ras* mRNA and protein (data not shown) were used for these studies. The parental IEC18 cell line was used as a control throughout these studies. Growth curves in monolayer culture to determine the exponential doubling time and the effects of TPA, colony formation in 0.3% agar and enzymatic assays for intracellular levels of DAG, were performed as previously described (Pai *et al*., 1991; Phan *et al*., 1991). Karyotype analysis were also preformed as previously described (Mitelman, 1992).

DNA and RNA analysis

DNA and RNA were isolated from the above cell lines as previously described (Jiang *et al*., 1992). Ten μg of genomic DNAs were digested to completion with the restriction enzymes BamHI, HindIII or EcoRI. The digested DNA was electrophoresed through a 1% agarose gel. The DNA was then transferred to Hybond-N hybridization membranes as previously described (Lu *et al*., 1988).

Total RNA was prepared from cultured cell lines by lysis in guanidinium thiocyanate and centrifugation through cesium chloride, as described (Lu *et al*., 1988). Ten μg of total RNA were electrophoresed through a 1% denaturing agarose gel containing 17% formaldehyde. RNA was transferred to Hybond-N hybridization membranes as described above.

The probes used for these studies were a gel purified 1.8 kb Bgl-2 cDNA fragment of Rb, and a 1.1 kb EcoRI cDNA fragment of cyclin D1. We found that it was essential to use probes that were devoid of plasmid sequences to avoid cross hybridization to plasmid sequences present in the DNA of the *ras*-transformed cells. Prehybridization was carried out for 2 h at 65°C in church buffer (Church and Gilbert, 1984). Fifty ng of the probes were labelled with [α-^{32}P]dCTP using the multiprime DNA labelling system (Amersham, Arlington Heights, IL), to a specific activity of 3×10^8 c.p.m./μl and added to the hybridization reaction at 65°C for 16–18 h. Following hybridization, the filters were washed in 2×SSC, 0.1% SDS, initially at room temperature for 20 min and subsequently for several additional washes at 65°C for 20 min each. The filters were then autoradiographed with Kodak XAR-5 X-ray film and Kodak 'lightning plus' intensifier screens for 1–7 days at −70°C. The autoradiograms were analysed by densitometric scanning.

Equivalent loading of lanes in both the Northern and Southern blots was confirmed by ethidium bromide staining and by repeating all assays at least three times.

Protein extraction and Western blotting

Exponentially growing cells were collected with a rubber policeman and washed three times in ice-cold PBS. The cell pellets were resuspended in lysis buffer (20 mM Tris-HCl pH 7.4, 2 mM EDTA, 2 mM EGTA, 6 mM β-mercaptoethanol, 1% NP-40, 0.1% SDS and 10 mM NaF, plus the protease inhibitors leupeptin 10 μg/ml, aprotonin 10 μg/ml and 0.1 mM phenylethylsulfonylfluoride). This suspension was sonicated on ice three times with a Sonifer Cell Disruptor, and centrifuged at 15 000 r.p.m. for 15 min to yield the soluble cell lysates. The lysates were quickly frozen and stored at $-80°C$ until use for further analysis. For Western blotting 50 μg of total cell lysates were subjected to 10% SDS–polyacrylamide gel electrophoresis. The proteins on these gels were transferred, using transfer buffer (25 mM Tris, 190 mM glycine, 10% methanol), to Immobilin -P membranes (Milipore, Bedford, MA) at 60 volt for 3 h. Membranes were blocked with blocking buffer (50 mM Tris, 200 mM NaCl, 0.2% triton X-100, 3% BSA) overnight at 4°C. The membranes were then incubated with a 1:1000 dilution of a polyclonal anti-human cyclin D antibody (UBI, Lane Placid, NY), or a monoclonal antibody to Rb (Pharmingen, San Diego, CA) for 60 min. After washing with washing buffer (blocking buffer without 3% BSA) for 1 h, horseradish peroxidase linked anti-rabbit donkey serum (1:5000) for cyclin D1, and anti-mouse sheep serum (1:2500) for Rb were added for 1 h. The membranes were then washed with washing buffer for 2 h and immune detection was performed using the ECL Western blotting detection system (Amersham, Arlington Heights, IL). The intensities of cyclin D1 and Rb proteins were quantitated by densitometric scanning. These experiments were also repeated at least three times and gave similar results.

Soft agar assay

Assays for growth in 0.3% Noble agar (Difco) were performed as previously described (Jiang *et al*., 1992). In brief, 8×10^4 cells were suspended in DMEM plus 5–10% FCS containing 0.3% agar and plated in triplicate in six well plates. After 3 weeks of growth, the cells were stained and the colonies counted by microscopy. These experiments were repeated twice.

Tumorigenicity assays

5×10^6 cells were injected subcutaneously into multiple sites in athymic mice. The animals were monitored for tumor formation every week and sacrificed 2 months later.

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VII. 4

Overexpression of Cyclin E Protein is Closely Related to The Mutator Phenotype of Colorectal Carcinoma

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ORIGINAL ARTICLE

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Overexpression of cyclin E protein is closely related to the mutator phenotype of colorectal carcinoma

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Abstract *Background and aims:* A subset of colorectal carcinomas are due to a deficiency in the DNA mismatch repair system. The molecular mechanisms of tumorigenesis in these tumors is not yet well understood. Deregulation of the cell cycle, specifically of the G_1 and S phases, is a hallmark of human cancers. Transition from the G_1 to the S phase is accelerated by increased cyclin E protein expression, and recent studies suggest that overexpression of cyclin E leads to chromosomal instability. The overexpression of cyclin E in a variety of human cancers, for example in colorectal, gastric, lung, breast, and kidney cancer, provides evidence that cyclin E plays a pivotal role in the cell cycle and replication. We examined whether the overexpression of cyclin E is related to the status of the mismatch repair system in colorectal carcinomas. *Patients and methods:* Frozen tumor samples and adjacent normal colon mucosa obtained from 100 patients were subjected to microsatellite analysis, RT-PCR, western blot analysis and immunohistochemistry. *Results:* High microsatellite instability was detected in 13 tumors, and in 10 of these (77%) cyclin E protein was overexpressed at least twofold compared to normal mucosa. In contrast, only 28 of the remaining 87 microsatellite stable tumors (32%) overexpressed cyclin E. Lower molecular weight cyclin E proteins were present in 7 of 87 microsatellite stable carcinoma (8%), compared to 7 cases exhibiting lower molecular weight isoforms of 13 MSI carcinoma (54%). *Conclusion:* Increased cyclin E protein expression and the appearance of lower molecular weight cyclin E proteins were significantly associated with MSI in colorectal tumors. The data indicate that increased and/or aberrant expression of cyclin E protein might contribute to the mutator phenotype of colorectal cancer.

Keywords Colorectal cancer · Cyclin E · Microsatellite instability · Mutator phenotype

Introduction

Colorectal cancer is one of the leading causes of death from cancer in the Western world. Up to 15% of colorectal carcinomas are due to a deficiency in the DNA mismatch repair (MMR) system, resulting from inactivation of at least five different genes: *hMSH2*, *hMLH1*, *PMS1*, *PMS2*, and *hMSH6* [1, 2, 3]. A hallmark of MMR deficiency is microsatellite instability (MSI). MSI is characterized by changes in length at simple repetitive nucleotide sequences caused by DNA replication errors. Colorectal carcinomas exhibiting MSI are preferably localized to the right hemicolon and are characterized by a poor differentiation and a favorable prognosis [4, 5, 6]. The molecular mechanisms of tumorigenesis in this subgroup of tumors is not yet well understood. In the colo-

rectum MSI is frequently associated with mutations in repetitive sequences of coding regions of the transforming growth factor β receptor II (TGFβ-RII) and the *BAX* gene, possibly interfering with the regulation of proliferation and/or apoptosis [7, 8]. Deregulation of the cell cycle, particularly of the G_1/S phase boundary, is considered as a central mechanism in human cancers [9]. The G_1/S phase transition is controlled by the precisely timed accumulation and degradation of cyclin E protein. An inappropriately elevated cyclin E protein level leads to the acceleration of the G_1/S transition [10]. Cellular abundance of cyclin E protein depends mainly on the ubiquitin mediated proteasome degradation pathway. In numerous studies tumor cells have revealed an overexpression of cyclin E compared to nontumorigenic cells. Evidence that cyclin E plays a role in tumorigenesis is provided by the overexpression of cyclin E in a variety of human cancers such as colorectal carcinomas, gastric, lung, breast, and kidney cancer [11, 12, 13, 14, 15]. In addition, the appearance of lower molecular weight (LMW) isoforms of the cyclin E protein was previously described in breast and colon cancer [14, 16].

To further clarify the process of colorectal carcinogenesis in MSI tumors we examined whether MSI is associated with characteristic alterations in cyclin E expression.

Materials and methods

Tissue specimens of primary colorectal carcinomas of various tumor stages and grades and of corresponding normal colonic mucosa were obtained from 100 patients who had undergone colorectal surgery. Normal colonic mucosa was dissected from the underlying muscular layer at the resection margin most distant from the tumor. Tumors were immediately frozen and stored in liquid nitrogen in the operating room and later subjected to DNA, RNA, and protein analysis. Hematoxylin and eosin (H&E) stains were routinely used to confirm the histological presence of adenocarcinoma.

MSI analysis

DNA was extracted from tumor samples and corresponding normal colonic mucosa using the QIAamp Tissue Kit (Qiagen). Polymerase chain reaction (PCR) was performed using a first-choice marker panel established by the International Collaborative Group on Hereditary Nonpolyposis Colorectal Cancer including two mononucleotide repeats (BAT25 and BAT26) and three dinucleotide repeats (D2S123, D5S346, and D17S250) as described by Boland et al. [17]. For each PCR reaction 100 ng genomic DNA was amplified using fluorescein-labeled primer. The PCR reaction consisted of 1×PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin, w/v), 200 µM of each deoxyribonucleoside triphosphate, 1 µM of each primer, and 0.5 U *Taq* polymerase (Pharmacia Biosynthesis) in a volume of 25 µl. PCR conditions consisted of denaturation at 94°C for 5 min, followed by 36 cycles of denaturation at 94°C for 45 s, primer annealing at temperatures ranging from 52°C to 59°C for 45 s, and primer extension at 72°C for 1 min.

PCR products were examined by the ALF DNA sequencer (Pharmacia, Biosynthesis). For ALF analysis the heat-denatured PCR samples were loaded onto a 6% denaturing polyacrylamide Reprogel (Pharmacia Biosynthesis) and electrophoresed at constant temperature (50°C).

MSI was scored as positive when altered and/or additional bands were present in at least two of five loci of the tumor as compared with normal DNA within the same patient. For confirmation of MSI the PCR assay was performed in duplicate.

RT-PCR analysis

Total RNA was isolated from tissue samples using Trizol reagent (Life Technologies). The first-strand cDNA was constructed from 5 µg total RNA by reverse transcription (RT) using the Superscript II Kit (Gibco-Brl). PCR products of cyclin E were amplified using the following primers: 5′-AATCGACAGGACGGCGAGGGAC-3′ and 5′-GGCAGTCAACATC-CAGGACACA-3′. The PCR conditions used for extension (72°C, 45 s), denaturing (94°C, 45 s), and annealing (58°C, 45 s) were repeated for 26 cycles.

Western blot analysis

Extracts of soluble cellular protein were lysed for 30 min at 4°C in lysis buffer [150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100] containing freshly added protease inhibitors (25 µg/ml aprotinin, 25 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). Samples of extracts containing 50 µg total protein were subjected to a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane by a semidry transfer (Bio-Rad). Protein quality and transfer efficiency was controlled by Ponceau staining of protein blots.

Cyclin E was detected using a monoclonal antibody raised against the COOH-terminal region of cyclin E (0.2 µmg/ml, clone HE12, Pharmingen) and visualized by horseradish peroxidase conjugated secondary antibodies and the enhanced chemiluminescence system (Amersham). The intensities of the bands were quantitated on a two-dimensional laser densitometer (Molecular Dynamics). The experiments were performed at least in duplicate.

Immunohistochemistry

Cryosections of 6 µm obtained from colorectal carcinomas and adjacent normal mucosa were fixed in acetone and treated with 1% H_2O_2 in 98% methanol. After being washed with phosphate-buffered solution and pretreated with 5% skimmed milk, slides were incubated at 20°C for 1 h with the anti-cyclin E monoclonal antibody (0.5 µmg/ml, clone HE12, Pharmingen). The sections were run through an avidin-biotin-peroxidase process (ABC kit) using biotinylated anti-mouse IgG (Vector). The immunoreaction was visualized by developing sections with DAB. Negative controls were included using a mouse IgG1 antibody against *Aspergillus niger* glucose oxidase (Dako).

Results

Analysis of microsatellite markers

To determine the rate of colorectal carcinomas exhibiting the mutator phenotype we performed MSI analysis. Of 100 colorectal carcinomas 13 tumors displayed MSI in at least two of five loci tested. None of the tumors demonstrated instability in only one marker. All MSI tu-

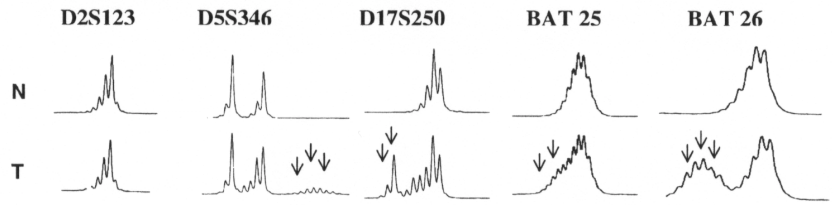

Fig. 1 Identification of a MSI tumor using an ALF sequencer. MSI loci were analyzed in normal colonic mucosa (*N*) and corresponding colorectal carcinomas (*T*). In colorectal carcinomas exhibiting MSI additional bands (*arrows*) were detected in at least two of five loci tested

Table 1 Evaluation of MSI; proportion of positive marker

Case	MSI marker					
	BAT25	BAT26	D2S123	D5S346	D17S250	
1	+	+	+	+	+	5/5
2	+	+	–	+	–	3/5
3	–	+	+	+	+	4/5
4	+	–	–	+	–	2/5
5	+	+	–	+	+	4/5
6	+	–	+	+	+	4/5
7	+	+	–	+	+	4/5
8	+	+	–	+	+	4/5
9	+	+	+	+	–	4/5
10	+	+	+	+	–	4/5
11	+	+	–	+	–	3/5
12	+	–	+	+	+	4/5
13	+	+	+	+	–	4/5
%	92	77	54	100	54	

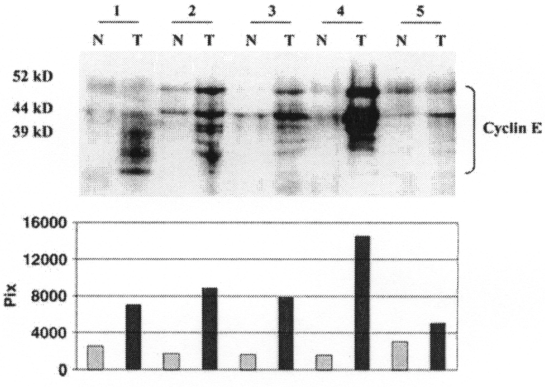

Fig. 2 Immunoblotting of cyclin E and two-dimensional laser densitometry in colorectal carcinoma (*T*) and adjacent normal mucosa (*N*). In colon carcinoma, overexpression of cyclin E was defined as an at least twofold expression compared to normal colonic mucosa (samples 1–4), lower molecular weight cyclin E proteins migrated between 30 and 39 kDa (samples 1, 2)

mors therefore exhibited high-frequency MSI. The results are shown in Table 1. Most MSI tumors (69%) revealed instability in four markers. In the tumors exhibiting MSI the mononucleotide repeat BAT25 and the dinucleotide repeat D5S346 were the most informative, being positive in 92% and 100% of the cases, respectively. A representative example of MSI analysis is illustrated in Fig. 1.

Protein expression of cyclin E in colorectal carcinomas

For characterization of MSI and MSS tumors regarding their cyclin E protein expression we performed western blot analysis on frozen tissue obtained from colorectal carcinomas and adjacent normal colonic mucosa. Immunoreactive bands were detectable at about 39, 44, and 52 kDa in tumor and normal mucosa specimens (Fig. 2). Intensity of the cyclin E immunoreactive protein bands was quantitated by two-dimensional laser densitometry. In tumors an overexpression of cyclin E was determined as an at least twofold increase in the intensities of the immunobands compared to adjacent normal mucosa (Fig. 2, samples 1–4). According to this, 38 out of 100 carcinomas revealed an overexpression of the cyclin E proteins. Of these cyclin E overexpressing tumors 14 samples exhibited additional lower molecular weight cyclin E bands, migrating to about 30 kDa (Fig. 2, samples 1, 2). In some of the tumors the lower molecular weight isoforms were even more abundant than the higher molecular weight proteins of cyclin E (Fig. 2, sample 1).

To further study the cellular distribution of cyclin E protein expression, H&E staining and immunohistochemistry were performed on 20 selected tumor specimens markedly overexpressing cyclin E and exhibiting lower molecular weight bands (Fig. 3a, b). Corresponding normal mucosa samples were used as controls. H&E staining revealed that the proportion of epithelial cells averaged 50% in normal mucosa and 70% in tumor specimens. In colorectal carcinomas overexpressing cyclin E immunostaining was confined to the nucleus of tumor cells (Fig. 3a) and was evenly distributed throughout the tumor. In normal mucosa there was only a scattered staining of the nuclei along the crypts (Fig. 3b). We did not observe cytoplasmic staining either in normal mucosa or in tumor tissue. Furthermore, cyclin E labeling was negative in stroma cells of either normal mucosa or tumor tissue. Expression levels of cyclin E as detected by immunohistochemistry paralleled the data obtained from western blotting.

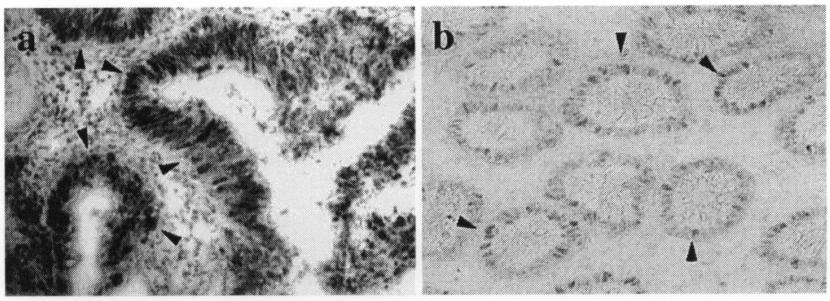

Fig. 3 Immunohistochemical staining for cyclin E in colorectal carcinoma (**a**) and in adjacent normal colonic mucosa (**b**), ×300. Positive staining (*arrowheads*) was restricted to the nuclei of colon carcinoma cells and normal colonic epithelial cells

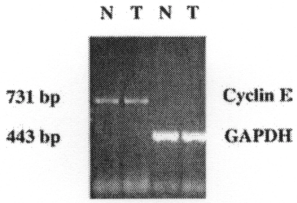

Fig. 4 Analysis of cyclin E mRNA expression by RT-PCR in a colorectal carcinoma (*T*) and adjacent normal colonic mucosa (*N*), GAPDH was used as an internal control

Expression of cyclin E mRNA in colorectal cancer

We further investigated whether the overexpression and the appearance of lower molecular weight proteins of cyclin E observed in a large proportion of colorectal carcinomas was due to an increased level or splicing of cyclin E mRNA. The 20 colorectal carcinomas previously analyzed by immunohistochemistry and adjacent normal mucosa samples were subjected to RT-PCR. Even in tumor samples that exhibited lower molecular weight bands we exclusively detected a single transcript of cyclin E at 731 bp. Levels of cyclin E mRNA were not significantly increased in colon tumors compared to normal mucosa and did not follow expression levels of cyclin E proteins. A representative example of RT-PCR analysis is shown in Fig. 4.

Relationship between MSI status and cyclin E protein expression profiles

We asked whether MSI tumors display characteristic profiles of cyclin E protein expression independently of tumor stage and grade. Interestingly, 10 of 13 MSI tumors (77%) displayed overexpression of cyclin E protein, and in 7 MSI tumors (54%) lower molecular weight protein bands were detected by cyclin E immunoblotting. In contrast, the proportion of MSS tumors exhibiting cyclin E overexpression was 32%. Only 7 of 87 MSS carcinomas (8%) presented lower molecular weight cyclin E bands. Thus the incidence of increased cyclin E expression and the appearance of lower molecular weight bands were both significantly ($P<.005$) related to the MSI status of colorectal carcinoma (Fig. 5). No correlation was found between cyclin E protein profiles and tumor stage and/or grade.

Discussion

In this study overexpression of the cyclin E protein was detected in 38% of colorectal carcinomas examined. There was no correlation between cyclin E protein expression and tumor stage or grade (data not shown). The cyclin E protein has been regarded as a crucial regulator of G_1/S phase transition. Overexpression of cyclin E protein has frequently been associated with various types of

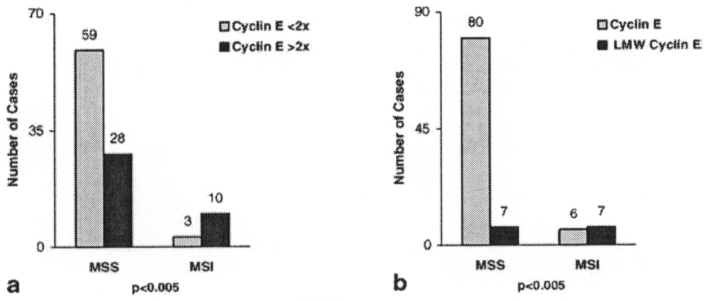

Fig. 5 **a** Cyclin E overexpression in colorectal carcinomas regarding to the MSI status, $P<0.005$ for association of cyclin E overexpression with MSI (Fisher's exact test). **b** Appearance of LMW cyclin E protein bands in colorectal carcinomas correlated to the MSI status, $P<0.005$ for association of cyclin E LMW bands with MSI (Fisher's exact test)

human cancer and considered as a marker of poor prognosis, for example in breast cancer [18]. In this context it is important to point out that the prognostic relevance of cyclin E expression seems to be tissue specific. In contrast to breast cancer, a decrease in cyclin E has been associated with the progression and a poor prognosis of bladder cancer [19]. In colon cancer no data have yet confirmed cyclin E as a prognostic marker. Although we observed no relationship between the histology of the tumor and the cyclin E expression. Yasui et al. [11] in a series of more than 400 patients reported an inverse correlation between the overexpression of cyclin E and the stages of colon carcinomas. This indicates that the overexpression of cyclin E might predict a favorable outcome in colon cancer.

High levels of cyclin E proteins in colorectal carcinomas were most likely not based on increased mRNA levels as demonstrated by RT-PCR analysis. Human cancer frequently displays alterations in the turnover of G_1 phase proteins, due to a dysfunction in the ubiquitin-mediated protein degradation pathway, as has been demonstrated for cyclin D1, p21 and p27 [20, 21, 22, 23, 24]. Since the ubiquitin-proteasome pathway also plays an important role in regulating the cellular abundance of cyclin E protein, increased levels of the cyclin E protein could be due to the inhibition of this protein degradation pathway [10, 25]. The detailed mechanisms of stabilization of cyclin E protein have not yet been completely elucidated. Possible mechanisms to achieve high protein levels of cyclin E are the mutation of cyclin E at an autophosphorylation site, for example threonin 380, or loss of function of the ubiquitin-protein ligases, for example cullins, resulting in an inhibition of the degradation pathway [10, 26].

Apart from the overexpression of the predominant cyclin E bands ranging from 39 to 52 kDa, there is a subset of colorectal carcinomas that display relative high amounts of faster migrating cyclin E proteins. The appearance of these lower molecular weight forms has been described in human breast and colorectal cancer [11, 16]. In breast cancer these lower molecular weight forms of cyclin E exhibit kinase activity and are probably not due to alternative splicing [27]. The authors suggested that lower molecular weight isoforms arise from posttranslational cleavage of cyclin E starting at the NH_2 terminus. It is important to note that this processing of the cyclin E protein occurs independently of the proteasome-mediated proteolytic pathway [27].

We have demonstrated for the first time that overexpression of cyclin E protein and the appearance of its lower molecular weight forms are significantly correlated with MSI in colorectal carcinomas. We examined whether there is a relationship between the overexpression of cyclin E and deficient DNA repair mechanisms? It has been shown that, as a regulatory subunit of CDK2 kinase, cyclin E participates in a number of processes at the G_1/S boundary. The cyclin E associated kinase activity is important for initiating and coordinating DNA synthesis and DNA replication. In this context, important functions of cyclin E are the inactivation of pRb, the participation in histone biosynthesis, in centrosome duplication, and in pre-mRNA splicing of genes involved into DNA synthesis [28, 29, 30, 31].

A possible explanation for our observation of a close relationship between the cyclin E overexpression and MSI is that cyclin E and/or its regulatory genes are targets of a defective MMR system similar to TGFβ-RII or Bax. Mutations of cyclin E and/or its regulatory genes, for example *cdk2*, *p27*, have not yet been reported in human cancer. In this context, it would be interesting to study genes that are involved in the cyclin E ubiquitination, such as *Cul1* and *Cul3* [32]. Alterations in the proteasome proteolytic system would at least provide evidence for the stabilization and overexpression of cyclin E but would not explain the presence of lower molecular weight cyclin E isoforms.

Another point of view is based on the observation that cyclin E is involved in the initiation of DNA replication by inactivating Rb [27], and that it participates in histone biosynthesis [30, 32] and in centrosome duplication [33]. Spruck et al. [34] demonstrated that constitutive overexpression of cyclin E induces chromosomal instability, thus interfering with the DNA duplication machinery. It is conceivable that overexpression of cyclin E causes an unscheduled DNA replication. The overexpression of cyclin E might therefore negatively interfere with the MMR system. There is a great variability in the penetrance of mutations in MMR genes regarding the mutator phenotype [3]. The cells that overexpress cyclin E may reinforce the phenotype of latent defective MMR systems such that it becomes detectable by MSI analysis. This would have to be tested, for example, by an in vitro system using constitutive cyclin E overexpression.

In conclusion, western blot analysis and immunohistochemistry demonstrated that a considerable proportion of colorectal carcinomas overexpresses cyclin E protein compared to normal colonic mucosa. In addition, a subset of colorectal carcinoma specimens overexpressing cyclin E display high levels of lower molecular weight cyclin E proteins that are barely detectable in normal mucosa as shown by western blot analysis. Results obtained from RT-PCR analysis revealed that increased cyclin E protein expression in colorectal cancer was most probably due to posttranslational modifications in cyclin E, and that there was no evidence for alternate splicing of cyclin E mRNA in colorectal carcinomas that could explain lower molecular weight cyclin E proteins.

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ANHANG

I Thesen

1. Adenokarzinome des Dünndarms weisen häufig eine Mutation im Codon 12 des K-ras-Protoonkogens auf.

2. Eine konstitutive Überexpression von K-ras in intestinalen Epithelzellen der Ratte führt zur mehr als fünffachen Überexpression von Cyclin D1 auf mRNA- und Proteinebene.

3. Die retrovirale Transduktion und über zwanzigfache Überexpression humaner Cyclin D1-cDNA in intestinalen Epithelzellen der Ratte beeinflußt weder die Morpholgie noch die Wachstumseigenschaften in vitro oder führt zur Tumorgenität dieser Zellen.

4. Eine erhöhte mRNA-Expression von Cyclin D1, verglichen mit normaler Kolonmukosa, ist in einem Anteil primärer kolorektaler unterschiedlicher Stadien und Differenzierungsgrade nachzuweisen.

5. Ebenso liegt häufig in kolorektalen Karzinomen aller Stadien und Differenzierungsgrade eine erhöhte Proteinexpression von Cyclin D1, verglichen mit normaler Kolonmukosa, vor.

6. Die mRNA- Expression von Cyclin E, verglichen mit normaler Kolonmukosa, ist in unterschiedlichen Stadien und Differenzierungsgraden primärer kolorektaler Karzinome erhöht.

7. Kolonkarzinome aller Stadien und Differenzierungsgrade zeigen, verglichen mit normaler Kolonmukosa und Kolonadenomen, häufig eine Überexpression, gelegentlich auch eine aberrante Expression von Cyclin E-Proteinen.

8. Es besteht kein direkter Zusammenhang zwischen der Cyclin E-Protein- und mRNA-Expression in primären kolorektalen Karzinomen.

9. Eine erhöhte Proteinexpression von Cyclin E ist daher zum Teil auf post-translationale Regulationvorgänge zurückzuführen.

10. Die relative Überexpression von Cyclin E-Proteinen ist mit dem malignen Phänotyp kolorektaler Tumoren assoziiert.

11. In primären Kolonkarzinomen besteht eine enge Korrelation zwischen einer erhöhten bzw. aberranten Cyclin E-Proteinexpression und dem Mutator-Phänotyp dieser Karzinome.

II Lebenslauf

Persönliche Angaben

geboren	26.05.1957 in Walsheim/Saar
Staatsangehörigkeit	deutsch

Universitäre Ausbildung

1976 – 1983	Studium der Humanmedizin Universität Würzburg, Lille (F), Homburg/Saar, Münster
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9/1984	Promotion zum Dr. med.

Klinisch-Wissenschaftlicher Werdegang

10/1984 – 4/1986	Wissenschaftlicher Assistent an der Klinik und Poliklinik für Allgemeinchirurgie der Ludwig-Maximilians-Universität München (Prof. Dr. Dr. h. c. G. Heberer)
5/1986 – 4/1987	Wissenschaftlicher Assistent an der Klinik für Herzchirugie der Ludwig-Maximilians-Universität München (Prof. Dr. Dr. h. c. W. Klinner)

5/1987 – 6/1992	Wissenschaftlicher Assistent an der Klinik und Poliklinik für Allgemeinchirurgie der Ludwig-Maximilians-Universität München (Prof. Dr. Dr. h. c. G. Heberer, ab 1989 Prof. Dr. Dr. h. c. F.-W. Schildberg)
7/1992 – 1/1995	Postdoctoral Research Fellow, Comprehensive Cancer Center, Columbia University New York (Dr. I. B. Weinstein)
1/1995 – 10/1995	Wissenschaftlicher Assistent an der Klinik und Poliklinik für Allgemeinchirurgie der Ludwig-Maximilians-Universität München (Prof. Dr. Dr. h. c. F.-W. Schildberg)
seit 10/1995	Wissenschaftlicher Mitarbeiter an der Klinik für Allgemein-, Viszeral- und Gefäßchirurgie der Martin-Luther-Universität Halle-Wittenberg (Prof. Dr. H. Dralle)
seit 8/1996	Oberarzt der Klinik

III Eidesstattliche Erklärung

Hiermit versichere ich an Eides statt, daß ich die vorliegende Habilitationsleistung selbständig verfaßt und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Zugleich erkläre ich, daß an keiner anderen Fakultät oder Universität ein Habilitationsverfahren derzeit anhängig ist bzw. jemals anhängig gewesen ist.

Halle, 26.03.2002 Dr. med. Thomas Sutter

IV Danksagung

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