# Untersuchungen zur aberranten DNA-Methylierung in humanen myeloischen Neoplasien mit Hilfe hochauflösender quantitativer Analysemethoden

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# Zusammenfassung

Die Methylierung von Cytosin an der Position C-5 im Dinukleotid CpG ist die einzige bekannte epigenetische Modifikation der DNA in Mammalia. CpG-Dinukleotide treten gehäuft als so genannte CpG-Inseln überwiegend im 5′-Bereich von Genen auf und sind in den meisten Fällen unmethyliert. Ein wichtiger Faktor im Verlauf einer neoplastischen Transformation ist die Hypermethylierung der CpG-Inseln von Tumor-Suppressor-Genen (TSG). Dies führt durch verschiedene Mechanismen zur Repression der betroffenen Gene.

Das Ziel der vorliegenden Arbeit war die Identifizierung und Charakterisierung potentieller Methylierungsmarker bei Patienten mit myelodysplastischen Syndromen (MDS) und akuter myeloischer Leukämie (AML). Für die Analysen wurden etablierte qualitative und quantitative Verfahren verwendet und darüber hinaus die hochauflösende, quantitative Pyrosequenzierung evaluiert und erfolgreich weiterentwickelt. Mit Hilfe dieser Verfahren konnten folgende Ergebnisse erzielt werden:

1. *SOCS-1* Hypermethylierung ist ein häufiges Ereignis in MDS und AML, korreliert mit dem Blastengehalt der Erkrankung und führt zu einer reduzierten Expression.
2. Eine Fehlinterpretation schwacher Methylierungssignale, welche bei bestimmten Genen auch in Normalgewebe zu finden sind, kann nur durch die Verwendung genspezifischer Schwellenwerte verhindert werden. Dies konnte exemplarisch für das *DAP-Kinase* Gen in MDS gezeigt werden. Die in der Literatur beschriebene aberrante Methylierung liegt quantitativ in einer Größenordnung, die auch in Kontrollbiopsien zu finden ist.
3. Eine aberrante Methylierung der Zellzyklusregulatoren $p21^{CIP1}$, $p27^{KIP1}$ und $p57^{KIP2}$ spielt ebenfalls keine wesentliche Rolle bei der Pathogenese von MDS und AML.
4. Die spezifische Detektion von Unterschieden im DNA-Methylierungsmuster, welche zwischen verschiedenen Stadien einer Erkrankung bestehen und somit ein großes prognostisches und diagnostisches Potential aufweisen, ist nur mit quantitativen Technologien möglich.
5. Aufgrund der Heterogenität der $p15^{INK4B}$ Methylierung in MDS und AML besitzen verschiedene Regionen dieser CpG-Insel ein signifikant unterschiedliches diskriminatorisches Potential. Diese krankheitsspezifischen Unterschiede lassen sich zuverlässig mittels Pyrosequenzierung identifizieren, da diese Methode eine quantitative Einzel-CpG-Analyse ermöglicht.

**Schlagwörter**: DNA-Hypermethylierung, myeloische Neoplasien, quantitative Methylierungsanalyse

# Astract

Methylation at position C-5 of cytosine in the dinucleotide CpG is the only epigenetic modification which is known in mammalia. CpG dinucleotides usually accumulate in so called CpG islands which are predominantly located in 5´ regions of genes and are generally unmethylated. One important factor in the progression of a neoplastic transformation is the hypermethylation of CpG islands of tumour suppressor genes (TSG) which leads through different mechanisms to the repression of the affected gene.

The aim of this thesis was the identification and characterisation of potential methylation markers in patients with myelodysplastic syndrome (MDS) and acute myeloid leukaemia (AML). The analyses were performed by using established qualitative and quantitative methodologies. Furthermore, Pyrosequencing™, a quantitative technology with a high resolution capacity was evaluated and successfully improved. With the aid of this technique the following results were achieved:

1. *SOCS-1* hypermethylation is a common event in MDS and AML. It correlates with the blast count of the disease and leads to a reduced expression.
2. Misinterpretation of weak methylation signals which can be found for some genes also in normal tissues, can be avoided by the application of gene-specific thresholds. This was exemplarily shown for the *DAP-kinase* gene in MDS. The aberrant methylation which was described in the literature showed a quantitative level which can be found in control biopsies as well.
3. Aberrant methylation of the cell cycle regulatory genes $p21^{CIP1}$, $p27^{KIP1}$ und $p57^{KIP2}$ also does not play a major role in the pathogenesis of MDS and AML.
4. The specific detection of varieties in the DNA methylation pattern which exist between different stages of a disease and therefore has a huge prognostic and diagnostic potential is solely possible with quantitative technologies.
5. Due to the heterogeneity of the $p15^{INK4B}$ methylation in MDS and AML there are different regions of this CpG island which show significant differences in their discriminatory potential. The identification of these disease-specific differences is only possible by using Pyrosequencing™ because this is the only method which enables the analysis of individual CpG sites.

**Keywords**: DNA hypermethylation, myeloid neoplasia, quantitative methylation analysis

# 1 Einleitung

## *1.1 Epigenetik*

Die Epigenetik (epi: über, auf) beschäftigt sich mit mitotisch und meiotisch vererbbaren Vorgängen, welche sich jenseits der konventionellen Genetik, also ohne Veränderungen der Nukleotidsequenz, abspielen und einen regulatorischen Einfluß auf die Genexpression ausüben. Sie geht also der Frage nach, welche Mechanismen dazu führen, dass die verschiedenen Zellen eines Organismus trotz ihrer identischen genetischen Ausstattung verschiedene Strukturen und Funktionen aufweisen. Zu diesen potentiell reversiblen und miteinander in Verbindung stehenden Prozessen gehören im Wesentlichen Veränderungen des Musters der DNA-Methylierung. Auch posttranslationale Modifikationen der Chromatinstruktur durch Methylierung, Azetylierung, Phosphorylierung und Ubiquitinierung von Histonen werden zu den epigenetischen Mechanismen gezählt. Ob Histon-Modifikationen stabil vererbt werden und diese Veränderungen somit alle Kriterien der Definition von Epigenetik erfüllen, konnte noch nicht abschließend geklärt werden[1].

## *1.2 DNA-Methylierung – ein epigenetischer Mechanismus*

Der weit verbreitete und evolutionär sehr alte Prozess der DNA-Methylierung ist bereits bei Prokaryonten zu finden[2]. Diese einzige bekannte post-synthetische Veränderung der DNA stellt beim Menschen die am besten untersuchte epigenetische Veränderung dar. Bei Mammalia versteht man unter DNA-Methylierung die kovalente Anheftung eines Methylrestes an die Base Cytosin. Der Transfer der Methylgruppe erfolgt von dem Methyldonor S-Adenosyl-L-Methionin (SAM) auf das Kohlenstoffatom Nr. 5 des Pyrimidinringes[3]. Dies geschieht symetrisch an beiden Strängen der palindromen Sequenz CpG (Cytosin-Phosphat-Guanin)[4]. Zwar gibt es Berichte über Methylierung in einem $C^{m}$pNpG und $C^{m}$C(A/T)GG Kontext[5-7], jedoch sind diese erst selten beschriebenen Methylierungereignisse aufgrund der Fehleranfälligkeit der zugrunde liegenden Methoden fraglich, und eine Bestätigung durch weitere Arbeiten bleibt abzuwarten.

### 1.2.1 Regulation der Methylierung

Der Vorgang der Methylierung erfolgt postreplikativ und wird durch DNA-Methyltransferasen (DNMT) vermittelt. Beim Menschen sind fünf verschiedene DNMT bekannt: DNMT 1, 2, 3A, 3B und 3L[3]. DNMT1 zeigt eine deutlich erhöhte Affinität für *hemi*-methylierte DNA, gegenüber unmethylierter DNA[8,9] und gilt daher im Wesentlichen als Erhaltungs-Methyltransferase, welche für die klonale Weitergabe des Methylierungsmusters während der DNA-Replikation verantwortlich ist. Für DNMT2 konnte anfänglich keine Methyltransferaseaktivität nachgewiesen werden[10]. Inzwischen ist bekannt, dass rekombinantes DNMT2 Protein eine schwache enzymatische Aktivität besitzt. Diese konnte sowohl *in vitro*[11] als auch *in vivo*[12] reproduzierbar nachgewiesen werden. Die biologische Funktion dieses Enzyms ist jedoch weiterhin unbekannt[10]. DNMT 3A und 3B gelten als *de novo* Methyltransferasen und sind für die Etablierung gewebespezifischer Methylierungsmuster zuständig[13,14]. DNMT3L stimuliert die katalytische Aktivität von DNMT 3A und 3B und ist hierdurch, zumindest bei Mäusen, essentiell an der Etablierung eines korrekten Methylierungsmusters beteiligt[15,16]. Eine eigene Methyltransferaseaktivität konnte für DNMT3L bislang nicht nachgewiesen werden.

Die Etablierung und Aufrechterhaltung eines korrekten Methylierungsmusters durch DNMT ist von elementarer Bedeutung. Bei DNMT-defizienten Mäusen kam es durch homozygote Inaktivierung bereits während der Embryogenese zu letalen Entwicklungsstörungen[17,18]. In Tumorzellinien konnte gezeigt werden, dass es durch das Ausschalten der beiden DNMT 1 und 3B zu einer Demethylierung vieler bekannter Tumor Suppressor Gene (TSG) kommt[19,20].

Über die Mechanismen der Demethylierung ist deutlich weniger bekannt. Eine Möglichkeit besteht in der passiven Reduktion von Methylierung durch die Deaktivierung der Methyltransferase-Aktivität während der DNA-Replikation[21]. Darüber hinaus wurde eine vollständige Demethylierung innerhalb weniger Stunden beobachtet, welche nicht mit einer Demethylierung während der Replikation vereinbar ist. Für eine aktive Demethylierung, unabhängig von der DNA-Replikation, konnten zwei verschiedene Mechanismen gezeigt werden[22]:

1. Austausch von 5-Methylcytosin gegen Cytosin. Hierbei wird 5-Methylcytosin durch 5-Methylcytosin-DNA-Glykosylase vom Phosphodiester-Rückgrat der DNA abgespalten und bei anschließender DNA-Reparatur durch Cytosin ersetzt[23,24].

2. Abspaltung der Methylgruppe durch Hydrolyse. Es konnte gezeigt werden, dass das klonierte Enzym 5-Methylcytosin-Demethylase in der Lage ist, 5-Methylcytosin zu Cytosin und Methanol zu hydrolysieren[25,26].

### 1.2.2 Vorkommen von Methylierung im Genom

Im menschlichen Genom liegen etwa 70 – 80% aller CpG-Stellen methyliert vor[1]. Der größte Anteil von ihnen ist in den sehr CpG-reichen Sequenzen der perizentromären Regionen zu finden[27]. Ein weiterer Teil der Methylierung ist in dem inaktivierten X-Chromosom der Frau und in autonomen DNA-Sequenzen zu finden. Hinzu kommt ein geringer Anteil am Methylierung in den Genen, welche dem „*Inprinting*“ (siehe unten) unterliegen. Bei pathologischen Veränderungen kommt es überdies zu einer Hypermethylierung von CpG-Inseln (siehe unten).

### 1.2.3 *„CpG-suppression“*

Der Anteil der existierenden CpG-Stellen hat sich im Laufe der Evolution deutlich reduziert. Verantwortlich hierfür ist die spontane Deaminierung von Cytosin[28,29]. Die Deaminierung von Cytosin führt zu Uracil. Hierdurch entsteht eine G:U Fehlpaarung, welche von der Uracil-DNA-Glycosylase effizient erkannt und repariert wird[30]. Eine Deaminierung von methyliertem Cytosin (5-mC) zu Thymin findet etwa 2- bis 3-mal häufiger statt[31] und die hierdurch entstehende G:T Fehlpaarung wird durch das Enzym wesentlich schlechter repariert[32]. Durch die deutlich erhöhte Mutationsfrequenz von 5-mC gegenüber anderen Nukleotiden kommt es zur *CpG-suppression*. Als Auswirkung hiervon liegt die beobachtete Häufigkeit des Dinukleotides CpG etwa 10 – 20fach unter dem Erwartungswert von 1/16[29,33].

### 1.2.4 *CpG-Inseln*

In einem Abstand von durchschnittlich 100 kb finden sich, über das gesamte Genom verstreut, Bereiche mit einer deutlich erhöhten CpG-Häufigkeit[34]. Diese CpG-Inseln haben eine Länge von 0,5 – 5 kb und machen etwa 1 – 2% des gesamten Genoms aus. Sie befinden sich meist im 5´-Bereich von Genen und sind bei etwa der Hälfte aller bekannten endogenen Säugergene zu finden[29,34,35]. Von Gardiner-Garden und Frommer[36] wurde eine CpG-Insel erstmals definiert als ein Bereich mit einer Länge von mehr als 200 bp, einem C+G Gehalt über 50% und einem Verhältnis der beobachteten zur erwarteten CpG-Häufigkeit von

mindestens 0,6. Da mit diesen Kriterien häufig nicht nur die eigentlichen CpG-Inseln, sondern auch repetitive *Alu*-Elemente identifiziert werden, wurden stringentere Parameter eingeführt: Länge ≥ 500 bp, C+G Gehalt ≥ 55% und beobachtete/erwartete CpG-Häufigkeit ≥ 0,65[37,38]. Die vergleichsweise hohe Dichte von CpG-Stellen in diesen Bereichen liegt darin begründet, dass diese in CpG-Inseln normalerweise unmethyliert vorliegen[39] und somit nicht der oben erwähnten sog. „*CpG-suppression"* unterliegen. Der Grund dafür, dass CpG-Inseln für gewöhnlich unmethyliert sind ist unbekannt. Eine mögliche Ursache hierfür ist der Schutz vor Methylierung durch eine aktive Transkription. Es wird vermutet, dass die Bindung von Transkriptionsfaktoren wie z.B. Sp1 an diese Regionen dazu führt, dass die CpG-Stellen nicht methyliert werden[40].

### 1.2.5 DNA Methylierung und Genexpression

Bei der Regulation der Transkription spielt die räumliche Anordnung der DNA eine wichtige Rolle. Eine Azetylierung der Histone bewirkt beispielsweise die Lockerung der Chromatinstruktur und macht die DNA für Transkriptionsfaktoren zugänglich. Durch ihr dynamisches Zusammenspiel mit Histon-modifizierenden Enzymen, welche den Azetylierungszustand der Histone regulieren, ist die DNA-Methylierung entscheidend an der Expressionskontrolle beteiligt[41]. DNA-Methylierung und Expression stehen in einem reziproken Verhältnis zueinander. Hierfür können zwei Mechanismen verantwortlich sein:

1. Es kommt zu einer direkten Hemmung der Bindung spezifischer Transkriptionsfaktoren an ihre Erkennungssequenz[42,43]. Diverse Transkriptionsfaktoren wie z.B. AP-2, cMyc/Myn, CREB, E2F und NF-κB binden an Sequenzen, welche CpG-Stellen enthalten, und für alle konnte die Inhibierung ihrer Bindung durch Methylierung gezeigt werden[44].

2. Starke Methylierung im Bereich von CpG-Inseln führt zur Rekrutierung von Methyl-CpG-Bindungsdomäne (MBD) Proteinen (siehe Abbildung 2 in Kapitel 1). Zu dieser Proteinfamilie zählen fünf gut charakterisierte Mitglieder: MBD1, MBD2, MBD3, MBD4 und Methyl-CpG-bindendes Protein 2 (MeCP2)[45]. Diese Proteine binden methylierungsspezifisch an die DNA[46,47] und assoziieren mit Histon-modifizierenden Proteinen wie Histondeazetylasen (HDAC)[48,49]. Die resultierende Deazetylierung der Histone bewirkt eine Kondensation des Chromatins[50]. Durch die geschlossene Struktur des Heterochromatins ist die DNA nicht mehr für Transkriptionsmaschinerie

zugänglich, und die von diesen Promotoren gesteuerten Gene können nicht mehr abgelesen werden[51]. Das Resultat ist ein Expressionsverlust der entsprechenden Gene[52].

Der Informationsgehalt des Genoms wird durch die DNA-Methylierung stark erhöht. Bei geschätzten 50 Millionen CpG-Stellen führt der Unterschied der beiden Zustände methyliert und unmethyliert zu $2^{50.000.000}$ denkbaren Kombinationsmöglichkeiten[53]. Selbst wenn nur ein kleiner Teil dieser möglichen Kombinationen an der Regulation der Genexpression beteiligt ist, wäre der Informationsgewinn durch Methylierung sehr hoch[54].

## *1.3 Funktionen von Methylierung*

### 1.3.1 Differenzierung von Zellen und Geweben

Methylierung spielt bereits während der Embryogenese eine wichtige Rolle bei der zelltyp-spezifischen Expression von Genen und der gewebespezifischen Differenzierung von Zellen[55]. Durch die methylierungsgesteuerte Aktivierung und Deaktivierung von Genen können Zellen mit unterschiedlichem Expressionsmuster entstehen, obwohl ihr genetisches Material völlig identisch ist. Dies ermöglicht die Entwicklung verschiedener Zellen bzw. Gewebe mit strukturellen und funktionellen Unterschieden.

### 1.3.2 *„Imprinting“* (Allel-spezifische Expression):

Bei nahezu allen Genen werden beide Allele exprimiert. Es gibt jedoch eine kleine Gruppe von Genen, welche allelspezifisch exprimiert werden[56]. Unter normalen Bedingungen bleibt bei diesen Genen entweder das mütterliche oder das väterliche Allel immer abgeschaltet. Der genaue Mechanismus dieses sog. „*Imprintig“* ist noch unklar. Es ist jedoch sicher, dass die DNA-Methylierung hierbei eine entscheidende Rolle spielt und die monoallelische Expression durch Methylierung von sog. „*differentially methylated regions*“ (DMR) gesteuert wird[56-58].

### 1.3.3 Inaktivierung des X-Chromosoms:

Eines der beiden weiblichen X-Chromosomen wird bereits sehr früh in der Embryonalentwicklung durch vollständige Methylierung deaktiviert[59]. Hierdurch wird die DNA äußerst kompakt und ist als Barr-Körperchen lichtmikroskopisch sichtbar.

### 1.3.4 Abwehr von parasitärer DNA

Es wird vermutet, dass der Grossteil der DNA-Methylierung im Säugergenom dem Schutz vor autonomen DNA-Sequenzen wie Transposons und Retroviren dient[27,60]. Durch ihre transkriptionshemmende Wirkung verhindert die Methylierung eine Aktivierung dieser „parasitären" Elemente. Darüber hinaus werden diese Sequenzen aufgrund der erhöhten Mutationsanfälligkeit von 5-mC zu T dauerhaft inaktiviert.

## *1.4 Zusammenhänge zwischen Methylierung und Krankheit*

In Tumorzellen findet man gleichzeitig eine globale Hypomethylierung[61,62] und eine regionenspezifische Hypermethylierung im Bereich der CpG-Inseln[63-65]. Die Mechanismen welche zu diesen beiden Zuständen führen sind unbekannt. Auch ob eine direkte Verbindung zwischen ihnen besteht ist unklar.

In einigen Arbeiten wurde eine Zunahme der Methylierung während des Alterns beschrieben[66-70]. Die Ursache hierfür ist nicht bekannt. Es wird angenommen, dass die Akkumulation von Methylierung (und vermutlich auch anderer epigenetischer und genetischer Defekte) im Laufe des Lebens mit der aberranten Hypermethylierung in Tumoren in Verbindung steht und diese zu dem erhöhten Krebsrisiko im Alter führen könnte[71].

### 1.4.1 Hypomethylierung

In Krebszellen ist die globale Methylierung gegenüber normalen Zellen sehr stark reduziert[72]. Zu einer Hypomethylierung kommt es vorwiegend in den normalerweise stark methylierten perizentromären Bereichen. Die Demethylierung von Satellitensequenzen kann zu homologen Rekombinationen und DNA-Brüchen führen. Hierdurch kommt es zu genomischer Instabilität, die zur Tumorentstehung und – progression beiträgt[65]. Bei einigen Krebsarten korreliert die Abnahme der Methylierung sogar mit der Aggressivität und dem Grad der Erkrankung[62,73].

Durch Hypomethylierung kommt es auch zur Aktivierung von Genen, welche in normalem Gewebe durch Hypermethylierung deaktiviert sind[74]. Dies betrifft vor allem Proto-Onkogene und Gene, welche dem *Imprinting* unterliegen. Proto-Onkogene haben wichtige Steuerungsfunktionen bei der Zellteilung. Ihre aberrante Aktivierung macht sie zu

Onkogenen. Diese verstärken die Zellproliferation und können hierdurch an der Entstehung von Tumoren beteiligt sein. Durch einen pathologischen Imprintingverlust (*loss of imprinting:* LOI) kommt es zu einer biallelischen Expression der betroffenen Gene. Die abnormale Expression beider Allele kann zu Tumoren und Missbildungen führen[75,76].

### 1.4.2 Hypermethylierung

Eine aberrante Hypermethylierung findet ausschließlich in CpG-Inseln statt. Diese Läsion ist bei Genen mit unterschiedlichen Funktionen zu finden: z.B. DNA-Reparatur, Zelldifferenzierung, Apoptose, Zellzykluskontrolle, Tumorsuppression, Signaltransduktion, Zelladhäsion. Bei den betroffenen Genen handelt es sich im Wesentlichen um bekannte oder mutmaßliche TSG deren Repression zu unkontrolliertem Zellwachstum führen kann[64,77].

Hypermethylierung kann zu einem Verlust von Genaktivität führen, welcher funktionell einem „*loss of heterozygosity*“ (LOH) im Sinne von Knudson´s „*Two-Hit*“-Hypothese[78] entspricht. Im Fall, dass beide Allele hypermethyliert sind, kann es auch unabhängig von einem zweiten Ereignis, in Form einer genetischen Veränderung, zur vollständigen Deaktivierung eines TSG kommen[54]. In vielen, vor allem hämatologischen Erkrankungen spielt Methylierung bei der Deaktivierung von TSG eine ähnlich bedeutende Rolle wie Mutationen und Deletionen[79].

### 1.4.3 *CpG Island Methylator Phenotype (CIMP)*

Bei der Analyse mehrerer Methylierungsmarker in kolorektalen Tumoren entdeckte die Gruppe um Jean-Pierre Issa die gleichzeitige Methylierung mehrerer Gene in einer Tumorprobe und nannte dieses Phänomen „*CpG Island Methylator Phenotype*“ (CIMP)[80,81]. Die untersuchten Karzinome wurden daraufhin in zwei Gruppen unterteilt: Häufig methyliert (≥ 3 Loci pro Tumor, Durchschnitt: 5,1) und selten methyliert (durchschnittlich 0,3 Loci pro Tumor). Ein CIMP konnte zwar in einigen Erkrankungen bestätigt werden, andere Studien fanden hingegen keinen Hinweis für dessen Existenz. Aus diesem Grund wird das Konzept kontrovers diskutiert[82,83].

### 1.4.4 Methylierung als therapeutisches Ziel

Aufgrund ihrer Reversibilität stellen Methylierungsveränderungen ein vielversprechendes therapeutisches Ziel dar. In mehreren klinischen Studien mit MDS- und AML-Patienten zeigten demethylierende Agenzien eine bessere Wirkung als die etablierte Standardtherapie[84,85]. Daraufhin wurden der sehr potente Methylierungsinhibitor 5-azacytidine (Vidaza) und dessen Analog 5-aza-2-deoxycytidine (Decitabine) im Jahr 2004 von der *Food and Drug Administration* (FDA) der USA als Medikament zur Behandlung aller MDS Subtypen zugelassen[86]. Ein weiteres kürzlich entwickeltes Cytidin-Analog, das Medikament Zebularine, wird zurzeit erprobt. Dieses Medikament zeichnet sich durch seine geringe Toxizität, eine hohe chemische Stabilität und die Möglichkeit zur oralen Applikation aus[87,88]. Ebenfalls sehr viel versprechend ist die zusätzliche Behandlung mit Deacetylaseinhibitoren wie Trichostain A[89]. Potentielle synergetische Effekte durch eine Kombination von Deacetylaseinhibitoren und Demethylierungsagenzien werden derzeit in klinischen Studien evaluiert[90,91]. Der große Nachteil dieser Medikamente ist jedoch ihre völlig unspezifische Wirkung. Es gibt Hinweise darauf, dass es bei langfristiger Behandlung, aufgrund der Hypomethylierung, zu genomischer Instabilität und somit zu Nebenwirkungen kommen könnte[92,93].

## *1.5 Myelodysplastische Syndrome (MDS)*

Myelodysplastische Syndrome bestehen aus einer heterogenen Gruppe klonaler Knochenmarkserkrankungen, welche eine inneffektive Hämatopoese und daraus resultierende periphere Zytopenien aufweisen. Die Inzidenz der MDS ist abhängig vom Alter und liegt durchschnittlich bei etwa 4/100000 Einwohnern pro Jahr. Sie können in jedem Alter auftreten, es sind jedoch vorwiegend Personen über 60 Jahre betroffen. Gekennzeichnet ist die Erkrankung durch dysplastische Veränderungen von mindestens einer myeloischen Zellreihe des meist hyperzellulären Knochenmarks. Bei vielen Patienten kommt es zu einer Transformation der Erkrankung in eine sekundäre AML. Das Risiko hierfür ist stark vom Subtyp der Erkrankung abhängig und liegt bei bis zu 30%.

Zur Erfassung der signifikanten prognostischen Unterschiede innerhalb der heterogenen Gruppe der MDS wurden 1982 durch die *French American British Cooperative Group* (FAB) definierte morphologische Kriterien festgelegt und die MDS in fünf Subgruppen

unterteilt: Refraktäre Anämie (RA), Refraktäre Anämie mit Ringsideroblasten (RARS), Refraktäre Anämie mit Exzess an Blasten (RAEB), Refraktäre Anämie mit Exzess an Blasten in Transformation (RAEB-t) und Chronisch Myelomonozytäre Leukämie (CMML). Diese Klassifikation wurde zur Einteilung der in der vorliegenden Arbeit analysierten Patientenproben verwendet. Die FAB-Klassifikation wurde 1999 von der Weltgesundheitsorganisation (WHO) durch eine neue Klassifikation ersetzt, welche aufgrund zusätzlicher Parameter und präziserer Einteilung einen höheren prognostischen Wert besitzen soll. Die prognostischen Vor- und Nachteile dieser beiden Klassifikationen werden allerdings immer noch kontrovers diskutiert[94-96].

## *1.6 Methoden zur Detektion von Methylierung*

Die erste Methode welche zur Untersuchung von DNA-Methylierung entwickelt wurde basiert auf dem Restriktionsverdau mit methylierungssensitiven Restriktionsenzymen, gefolgt von einer Southern-Blot-Hybridisierung[97,98]. Diese Methode hat diverse Nachteile: Es wird eine große Menge hochmolekularer DNA benötigt, man erhält lediglich Informationen über einzelne, in der Erkennungssequenz der Enzyme befindliche CpG-Stellen, und sie ist anfällig für falsch positive Ergebnisse durch unvollständigen DNA-Verdau. Im Laufe der Zeit wurden viele deutlich bessere Methoden entwickelt, welche sowohl die Analyse von globaler wie auch von genspezifischer Methylierung ermöglichen[99,100]. Die Methoden lassen sich grundsätzlich in zwei Gruppen unterteilen. Eine Gruppe basiert auf der oben beschriebenen Verwendung von methylierungssensitiven Restriktionsenzymen und dient hauptsächlich der genomweiten Methylierungsanalyse. Die andere, weitaus größere Gruppe, basiert auf der Veränderung der DNA durch Natrium-Bisulfit[101] und wird weitestgehend für die Methylierungsanalyse von CpG-Inseln verwendet. Die Behandlung der DNA mit Natrium-Bisulfit wandelt die schwer zu analysierende epigenetische Information, methyliertes Cytosin oder unmethyliertes Cytosin, in einen Unterschied der primären Sequenz um (5-mC → C und C → U → T (post PCR)). Dieser Unterschied kann anschließend mit einer Vielfalt von Methoden untersucht werden. Im Folgenden sollen die in dieser Arbeit zur Methylierungsanalyse verwendeten Methoden kurz vorgestellt werden.

### 1.6.1 Methylierungs Spezifische PCR (MSP)

Bei der MSP[102] handelt es sich um die am weitesten verbreitete Methode zur Untersuchung des Methylierungsstatus von bestimmten CpG-Stellen in CpG-Inseln. Diese qualitative Methode basiert auf einer PCR-basierten Diskrimminierung zwischen methylierter und unmethylierter DNA nach Natrium-Bisulfit-Behandlung. Mit jeder zu untersuchenden Probe werden zwei unabhängige PCR-Reaktionen mit zwei verschiedenen Primerpaaren durchgeführt. Es wird ein Primerpaar verwendet, welches spezifisch nur methylierte DNA (M-DNA) amplifiziert (M-Primer) und ein zweites, welches spezifisch nur unmethylierte DNA (U-DNA) amplifiziert (U-Primer). Beide PCR-Produkte werden anschließend mittels Gelelektrophorese und Ethidiumbromidfärbung visualisiert.

### 1.6.2 Methy Light (ML)

Beim ML-Assay[103] handelt es sich um eine zur Methylierungsanalyse entwickelte Version der quantitativen *real-time* PCR (siehe Abbildung 1). Nach der Behandlung der DNA mit Natrium-Bisulfit wird eine PCR mit zwei methylierungsspezifischen Primern und einer zwischen ihnen lokalisierten Sonde durchgeführt. Die ebenfalls methylierungsspezifische Sonde ist 5´ mit einem fluoreszierenden *Reporter*-Farbstoff (z.B. FAM) und 3´ mit einem *Quencher*-Farbstoff (z.B. TAMRA) markiert. Zwischen den Farbstoffen besteht ein Fluoreszenz-Resonanz-Energietransfer (FRET). Nach der Anlagerung an das PCR-Produkt wird die Sonde durch die 5´ → 3´ Exonukleaseaktivität der *Taq*-Polymerase abgebaut. Es kommt zur räumlichen Trennung der beiden Farbstoffe und der hieraus resultierende Verlust des FRET führt zum Freisetzen des Fluoreszenzsignals. Die Fluoreszenz ist proportional zu der Menge an generiertem PCR-Produkt und wird bei jedem PCR-Zyklus gemessen. Um eine relative Quantifizierung zu ermöglichen, wird parallel eine interne Kontrolle in Form eines Referenz-Gens gemessen. Die Werte des PCR-Zyklus, in welchem der Detektions-Schwellenwert (*Threshold Cycle*: $C_T$) überschritten wird, die Fluoreszenz also erstmalig signifikant über die Hintergrund-Fluoreszenz ansteigt und die Reaktion in die exponentielle Phase übergeht, werden miteinander verrechnet ($C_T$(Zielgen) – $C_T$(Referenzgen) = $\Delta C_T$). Der durch diese so genannte Normalisierung errechnete $\Delta C_T$-Wert stellt den ursprünglichen relativen Methylierungslevel der Probe dar. Mit Hilfe einer Verdünnungsreihe aus methylierter und unmethylierter DNA wird eine Eichgerade erstellt, welche als Referenz für die Umrechnung von $\Delta C_T$-Werten in prozentuale Methylierung dient.

### 1.6.3 Pyrosequenzierung

Eine neue, sehr vielversprechende Methode zur Methylierungsanalyse ist die Pyrosequenzierung (siehe Abbildung 2). Hierbei handelt es sich um eine *real-time* Technologie, welche auf dem Prinzip der DNA-Sequenzierung während der Synthese basiert[104,105]. Im ersten Schritt wird ein PCR-Produkt mit methylierungsinsensitiven Primern (i-Primer) hergestellt. Nach Denaturierung des PCR-Produktes wird vor die zu analysierende Region ein Sequenzierprimer hybridisiert. Zu diesem Reaktionsansatz werden in einer vorgegebenen Reihenfolge Nukleotide hinzugefügt. Ist das zugegebene Nukleotid zum *Template* komplementär, so wird es von der DNA-Polymerase eingebaut, anderenfalls wird es enzymatisch abgebaut. Ein Einbau führt zur Freisetzung von Pyrophosphat (PPi) in einer Menge, welche äquimolar zur Anzahl der eingebauten Nukleotide ist. Das PPi wird durch eine Enzym-Kaskade in ein detektierbares Fluoreszenzsignal umgewandelt. Dieses Fluoreszenzsignal wird von der Firmensoftware in ein Pyrogramm umgewandelt, welches die Art und die Menge der eingebauten Nukleotide darstellt. Ursprünglich wurde die Methode zur Analyse von Einzelnukleotidpolymorphismen (*single nucleotid polymorphism* (SNP)) entwickelt. Da sich der Unterschied zwischen methyliertem und unmethyliertem Cytosin durch Behandlung mit Natrium-Bisulfit in einen SNP vom C/T Typ umwandeln lässt, eignet sich diese Methode auch zur Methylierungsanalyse[106-113]. Pyrosequenzierung ermöglicht zurzeit als einzige Methode die präzise Charakterisierung mehrerer einzelner CpG-Stellen und deren gleichzeitige Quantifizierung.

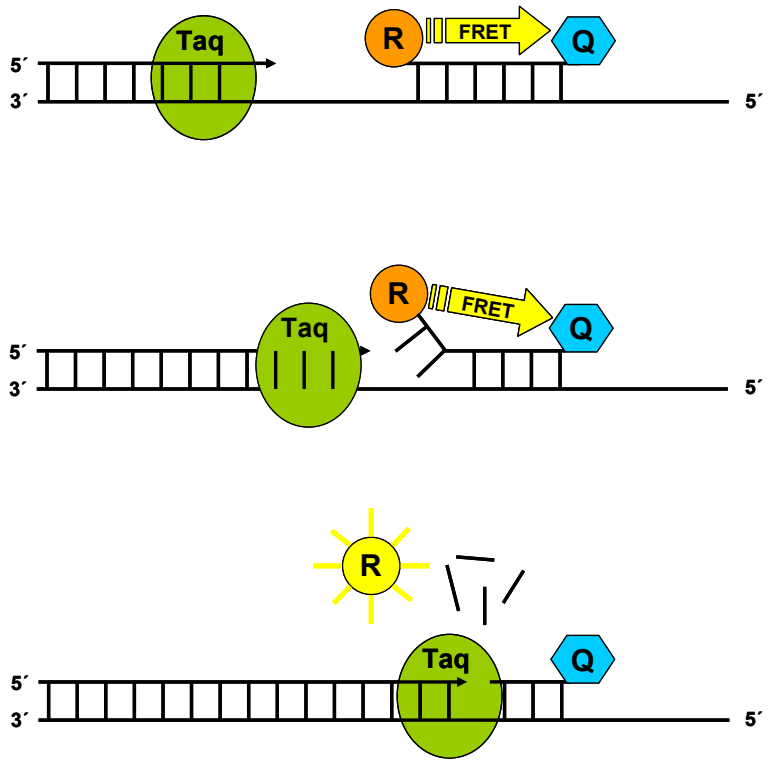


**Abbildung 1: Prinzip der *real-time* PCR mit Hydrolysesonden**

Die zwischen zwei PCR-Primern lokalisierte Sonde trägt zwei flankierende Fluoreszenzfarbstoffe. Aufgrund der räumlichen Nähe besteht ein Fluoreszenz-Resonanz-Energietransfer (FRET) vom *Reporter*-Farbstoff (R) auf den *Quencher*-Farbstoff (Q). Durch die 5´ → 3´ Exonukleaseaktivität der Taq-Polymerase wird die Sonde abgebaut. Die räumliche Trennung der Farbstoffe führt zum Verlust des FRET. Das freigesetzte Fluoreszenzsignal des *Reporter*-Farbstoffes ist proportional zur Menge des generierten PCR-Produktes.

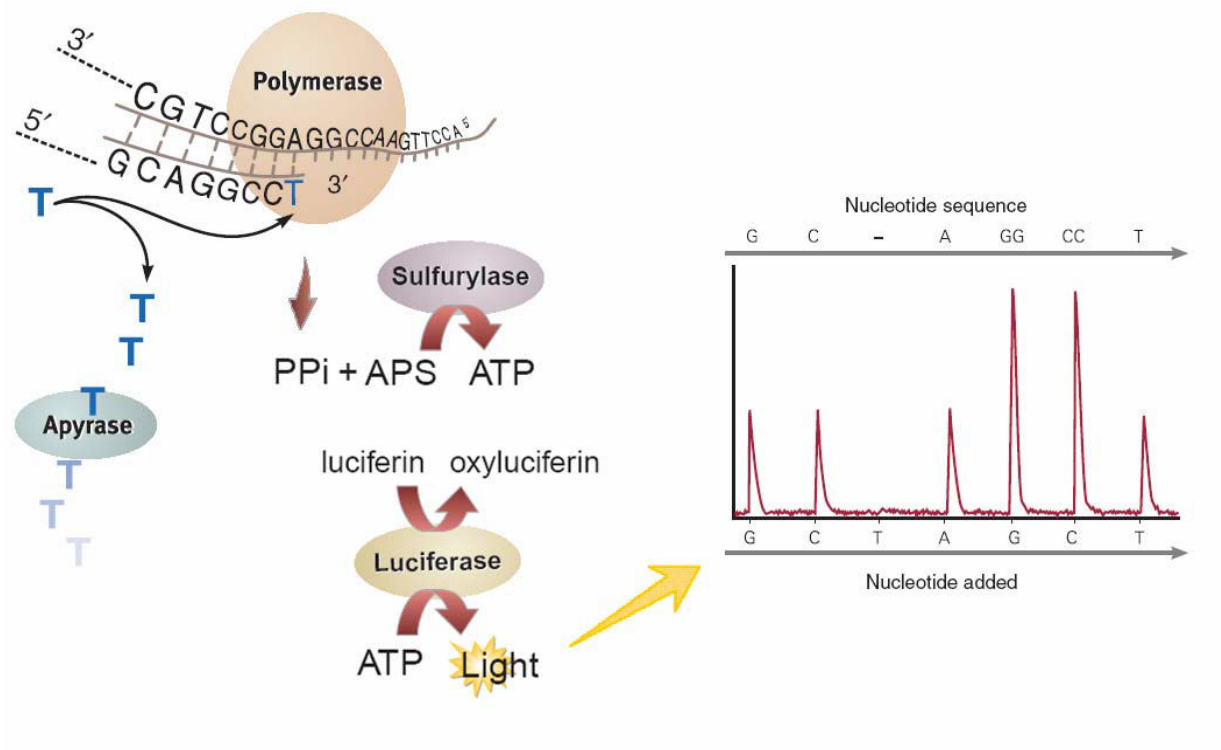


**Abbildung 2: Prinzip der Pyrosequenzierung**

Der Einbau eines Nukleotides führt zur Freisetzung von Pyrophosphat (PPi), welches in Anwesenheit von Adenosin 5'-Phosphosulphat (APS) durch ATP-Sulphurylase in Adenosin Triphosphat (ATP) umgewandelt wird. In Gegenwart von ATP wird Luziferin durch Luziferase in Oxyluziferin umgewandelt. Hierdurch entsteht detektierbares Licht, welches als *Peak* in einem Pyrogramm dargestellt wird. Die Höhe dieses *Peaks* ist proportional zur Menge der eingebauten Nukleotide. Überschüssige Nukleotide werden durch Apyrase abgebaut, bevor das nächste Nukleotid zugegeben wird (Abbildung nach www.pyrosequencing.com).

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# 3 Ziele der Arbeit

Das Ziel der vorliegenden Arbeit war es, den Methylierungsstatus von Tumor Suppressor Genen zu untersuchen, deren Hypermethylierung bei der Entstehung und Progression von myeloischen Neoplasien eine wichtige Rolle spielen könnte.

Durch Literaturrecherche (siehe Kapitel 1) wurden einige viel versprechende Kandidaten ermittelt. In einigen Fällen lagen widersprüchliche und fragliche qualitative Daten vor, welche mittels quantitativer Analyse geklärt werden sollten. Bei den ausgewählten Genen handelt es sich um zyklinabhängige Kinaseinhibitoren der INK4-Familie ($p15^{INK4B}$) und der CIP/KIP-Familie ($p21^{CIP1}$, $p27^{KIP1}$, und $p57^{KIP2}$), welche bei der Zellzyklusregulation eine wesentliche Rolle spielen, eine pro-apoptotische Serin/Threonin Kinase (*Death Associated Protein Kinase*: *DAP-Kinase*) sowie einen Negativ-Regualtor einiger Zytokin-vermittelter Signalwege (*Suppressor Of Cytokine Signalling 1*: *SOCS-1*), welcher einen Einfluß auf den JAK/STAT Weg hat und hierdurch regulatorisch an der Übertragung extrazellulärer Signale ins Zellinnere beteiligt ist. Für alle diese Gene ist eine Tumor-Suppressor-Funktion bekannt.

Für Gene, bei denen eine aberrante Methylierung identifiziert werden konnte, sollte versucht werden, durch eine quantitative Charakterisierung und die Festlegung von genspezifischen Schwellenwerten, krankheitsspezifische Methylierungsunterschiede zuverlässig nachzuweisen. Darüber hinaus sollte untersucht werden, ob bei einem Gen mit einem heterogenen Methylierungsmuster Regionen mit signifikanten, krankheitsspezifischen Unterschieden zu finden sind. Um die für diese Fragestellung notwendige quantitative Einzel-CpG-Analyse einer gesamten CpG-Insel zu ermöglichen, sollte die derzeit einzige hierfür geeignete Methode, die Pyrosequenzierung, technisch weiterentwickelt und evaluiert werden. Das heterogen methylierte $p15^{INK4B}$ Gen sollte mit dieser hochauflösenden Technik auf das Vorhandensein von Regionen mit unterschiedlichem diskriminatorischem Potential innerhalb seiner CpG-Insel überprüft werden.

Durch diese Untersuchungen sollte ein besseres Verständnis über die Bedeutung der aberranten Methylierung der untersuchten Gene in MDS und AML gewonnen und deren prognostischer und diagnostischer Wert evaluiert werden.

MDS wurden als Modellsystem gewählt, da sie die Möglichkeit bieten, Korrelationen zwischen Methylierungsstatus und dem Verlauf und Transformationsrisiko der Krankheit zu untersuchen. Obwohl das Transformationsrisiko von MDS in eine sekundäre AML bei bis zu 30% liegt, beschränkt sich der Wissensstand über aberrante Methylierung auf wenige Gene und ist damit deutlich geringer als der bezüglich AML. Darüber hinaus stellt Hypermethylierung gerade bei MDS ein sehr interessantes therapeutisches Ziel dar. Erst kürzlich wurden zwei demethylierende Medikamente von der FDA zur Behandlung von MDS-Patienten zugelassen (siehe oben).

# Kapitel 1

## Role of epigenetic changes in hematological malignancies

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*Role of epigenetic changes in hematological malignancies.*
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**Abstract**
Inactivation of tumour suppressor genes is an important event contributing to the development of neoplasia. In addition to the classical genetic mechanisms of deletion or inactivating point mutations growth regulatory genes can be functionally inactivated without alterations of the primary sequence by methylation of cytosine residues in the promoter regions of the genes.
After introducing epigenetic phenomena in general and the molecular basis of DNA methylation in more detail, this review will present the broad spectrum of alterations in DNA methylation patterns found in hematopoietic malignancies. In addition, the implications for therapy and prognosis will be discussed.

**Keywords**
Epigenetics, DNA methylation, DNA methyltransferases (DNMT), DNMT inhibitors
Leukemia, Lymphoma, Myelodysplastic syndrome (MDS)

**"Epigenetics"**

The term "epigenetics" has already been used for quite a long time and originally referred to changes in gene expression during development [87]. A current, more focused definition refers to "modifications in gene expression that are brought about by heritable, but potentially reversible changes in chromatin structure and/or DNA methylation" [71]. This definition already points at the two epigenetic systems that affect animal development and fulfil the criterion of heritability: the Polycomb-trithorax group (Pc-G/trx) of transcriptional repressors and their antagonists and the methylation of the DNA base cytosine. Whether the covalent modification of histones by e.g. methylation or acetylation, is stably inherited is still a matter of debate.

The Polycomb group (Pc-G) of transcriptional repressors form quite large multiprotein complexes, which are involved in the maintenance of a closed chromatin structure thereby inhibiting transcription of target genes. The best known and most intensively studied target genes are the *Hox* genes coding for transcription factors important in the formation of the anteroposterior body axis [105]. The activities of the Polycomb group repressors are specifically antagonized by a family of transcriptional activators named "trithorax". For a recent comprehensive review about the role of several mammalian Pc-G members in tumorigenesis, see [79].

This review will concentrate on the role of alterations in DNA methylation as the most important and best analysed epigenetic mechanism in human development and disease. It does not cover a large body of data obtained by analysing established cell lines, because there exist huge differences in methylation frequencies and patterns between primary tumours and cell lines [6, 135]. Also experimental data obtained from animal models are mentioned only occasionally.

Since the DNA methylation field has almost exploded in recent years producing a tidal wave of publications, a complete coverage is not possible and we apologise for all omissions necessary for reasons of comprehensibility.

**DNA methylation – some basic facts**

DNA methylation in higher eukaryotes affects only the base cytosine which becomes covalently modified at the carbon atom no. 5 (Fig. 1). This modification takes place only when the cytosine is followed by a guanine base, in other words, only the dinucleotide CpG is methylated. Methylated cytosine is a target for point mutations because deamination of 5’-methyl-cytosine leads to the formation of the naturally occurring DNA base thymidine,

which cannot be recognised as mutated by the DNA repair machinery of the cell. Since deamination is constantly taking place under physiological conditions, albeit with a low frequency, there is a selective pressure to reduce the number of methylated cytosine bases throughout the genome. In the mammalian genome, this has led to the progressive depletion of the CpG dinucleotides during evolution. CpG appears approximately only once per 80 dinucleotides, instead of once per 16 dinucleotides as expected from a random distribution. Only the physiologically unmethylated CpG sites in the promoter region and first exons of transcribed genes remained unaltered because active transcription seems to protect CpG sites against methylation [30]. These mostly unmethylated CpG-rich stretches of DNA form the so-called "CpG islands" in the "sea" of the CpG depleted genome. These CpG islands comprise 1-2% of the genome and are characterized by an overall CG-content of more than 55% and an approximately five times more frequent occurrence of the dinucleotide CpG [21, 61].

A notable exception are the constitutively methylated CpG islands found in imprinted genes [142] and on the inactive X-chromosome in females, which is nearly completely methylated [156].

DNA methylation leads in concert with histone modification, namely histone deacetylation, to the formation of a dense, more compact chromatin structure, which reduces the accessibility of promoter regions for the basal transcription machinery and accessory factors, thereby inhibiting gene expression (see Figure 2).

**Detection of CpG-Methylation – Methodological considerations**

Since the different methodologies for detection of cytosine methylation have recently been reviewed comprehensively by Fraga and Esteller [52], only some short comments are given here.

Nearly all assays in use today can be divided into two groups: restriction enzyme-based and bisulfite treatment-based. The former employs the inhibition of certain restriction enzymes by methylation of their recognition sites as an indicator for the presence of methylation. The latter translates the epigenetic information of cytosine methylation in primary sequence differences by converting unmethylated cytosine to uracil (which is subsequently amplified as thymidine) whereas methylated cytosine remains unaltered (Fig. 3 A). The bisulfite converted genomic DNA can be analysed by a wide variety of PCR-based methods of which direct sequencing of the PCR products or sequencing of individual PCR product clones

gives the most detailed information about the methylation pattern in the CpG islands under study. For further information, see [29]

Several protocols have been published for the analysis of minute amounts of DNA [81, 113], and also for the analysis of DNA isolated from archival formalin-fixed paraffin-embedded biopsies [5, 83, 91, 157], which now allow the retrospective correlation of molecular findings with the morphological diagnosis, the response to treatment and the clinical outcome.

Since under many circumstances the purely qualitative detection of aberrant methylation is not sufficient and may mask important quantitative differences, several groups developed methylation assays providing quantitative information, most of them based on real-time PCR technology [41, 65, 91, 97, 121].

Also micro array-based methods with the potential for high-throughput analysis of hundreds or even thousands of methylation sites in parallel have been described in first proof-of-principal publications [1, 13, 63, 73, 180].

**Hypomethylation in hematological malignancies**

In parallel to the well analysed gene-specific hypermethylation (see below), a decrease in methylation also can be observed during the process of malignant transformation (for a recent review see [43]). This decrease affects primarily repetitive sequences but also single-copy genes. Due to the large size of the repetitive sequences displaying reduced levels of methylation, in comparison to the single-copy genes showing hypermethylation, the overall methylcytosine content of most tumour cells is significantly reduced. Despite the fact that hypomethylation in tumour cells was described well before single-copy gene hypermethylation was discovered [50, 56], the latter phenomenon is analysed in much more detail and the functional consequences are far better understood.

From the studies addressing the occurrence and significance of hypomethylation in malignant neoplasia only a small fraction deals with hematopoietic malignancies.

Reduced overall methylcytosine content and hypomethylation of highly repetitive LINE-1 sequences was demonstrated for B-cell chronic lymphocytic leukaemia [33, 171]. For this entity also a reduced methylation level of the *bcl-2* gene was reported [68]. But this reduced methylation level did not correlate with the protein expression levels as assessed by immunoblotting. In contrast to these findings, the hypomethylation of the *MDR1* gene found in acute myeloid leukaemia samples correlated very well with reduced expression as assessed by quantitative reverse transcription-polymerase chain reaction. This might

contribute to P-glycoprotein-mediated multidrug resistance in AML patients [124]. More recently, activation of *TCL1* oncogene transcription by reduced methylation was described for a variety of mature B-cell lymphomas including B-cell chronic lymphocytic leukaemia and Burkitt's lymphoma [182]. Gene-specific demethylation has also been described for c-*myc* [167], *myeloperoxidase* [103] and *ornithine decarboxylase* [96].

In all studies mentioned above, the methylation status of single or multiple CpG dinucleotides was analysed by using methylation-sensitive restriction enzymes followed by Southern blotting or PCR.

In addition to the ectopic expression of hypomethylated single-copy genes, hypomethylation contributes to the development of chromosomal instability [92] which subsequently led to the development of aggressive T cell lymphomas in a mouse model [62].

**Hypermethylation**

The list of genes for which an aberrant methylation in hematopoietic malignancies is reported in the literature is steadily growing. Therefore, the following compilation of genes affected by DNA methylation cannot be exhaustive.

In Table 1 only for those studies which analysed more than 20 cases of a given disease are the results shown. In the column "Additional References" publications are listed which report the analysis of less than 20 cases.

Table 2 contains genes for which in only a single publication more than 20 cases have been analysed.

In addition to the genes listed in Table 2, an aberrant methylation has been reported for dCK [38], EphA3 [40] and SYK [66]. In these studies the methylation status for each entity was analyzed in less than 20 cases.

The functional consequences of gene hypermethylation are in most instances not adequately analyzed. Only a subset of studies correlates methylation data with measurement of gene expression. In Table 4 all studies analyzing methylation and expression in more than 20 samples are listed. Expression data for various genes studied in smaller patient cohorts are presented in the following publications: [15, 23, 47, 58, 75, 94, 102, 116, 130, 136, 153, 166, 169]. Most studies employ Reverse Transcriptase-PCR or immunohistochemistry, whereas only very few used Western Blot analysis (e.g. [136]).

For most genes inactivcated by hypermethylation in haematological malignancies a causative role in lymphomagenesis or leukemogenesis has not yet been demonstrated

unequivocally. Some hypermethylated genes may just function as a marker for a regionally hypermethylated chromosomal area, like calcitonin on chromosome 11p15 [17].

*DAP-Kinase*

The "death associated protein" (DAP) kinase belongs to a family of at least five proapoptotic serine/threonine kinases [126]. It was first identified by a technical knockout screen based on random inactivation of genes by expression of an antisense cDNA library followed by selection of clones that survived the continuous apoptotic stimulus of IFN-γ [36]. The DAP-Kinase protein is involved in mediating apoptosis in response to oncogene induced hyperproliferation and in response to detachment from extracellular matrix. The gene shows epigenetic inactivation by promoter hypermethylation in a wide variety of epithelial and hematopoietic malignancies.

Hypermethylation of this gene is a frequent event in multiple myeloma and found much more often in B-cell lymphomas as compared to T-cell lymphomas (see Table 1). With the exception of adult AML, methylation of the *DAP kinase* gene is quite rare in leukaemias.

*HIC-1*

The *HIC-1* gene ("hypermethylated in cancer") encodes for a member of the ZiN (zinc finger N-terminal) or Poz (Pox/zinc finger) family of zinc finger containing transcription factors. It was identified screening for hypermethylated regions of the genome which might harbour candidate tumour suppressor genes [172]. The gene is located on chromosome 17p13.3, a region that is frequently deleted independently from the more centromerically located *p53* gene in several tumour types like brain or breast tumour [106, 172]. Subsequently, the *HIC-1* gene was shown to be hypermethylated in a wide variety of epithelial and hematopoietic malignancies [55, 78, 117]. Issa et al. [78] described a preferential epigenetic inactivation in acute lymphoblastic leukaemia in contrast to acute myeloid leukaemia.

More recently, Baylin and colleagues [27] identified *HIC-1* as the first candidate tumour suppressor that is transcriptionally silenced, rather than mutated, and that acts as a tumour suppressor in mice. Interestingly, mice heterozygous for the *HIC-1* gene (HIC-1+/-) displayed a remarkably gender–dependent tumour spectrum in that male heterozygotes developed carcinomas at an increased frequency, whereas female heterozygotes mostly developed lymphomas and sarcomas. The underlying molecular mechanisms for these findings are not yet defined.

*$p15^{INK4b}$, $p16^{INK4A}$*

The two cell cycle regulatory genes *$p15^{INK4b}$* and *$p16^{INK4a}$* are by far the best studied examples concerning epigenetic inactivation in haematological malignancies. Both genes are located on chromosome 9p21 and code for inhibitors of the cyclin-dependent kinase 4 and 6 (INhibitor of CDK4 and CDK6), thereby interfering with cell cycle progression at the G1/S transition [131]. The *$p15^{INK4b}$* gene is induced in response to transforming growth factor beta (TGF-β) treatment by direct activation of transcription [69].

Whereas homozygous deletions of either both *$p16^{INK4a}$* and *$p15^{INK4b}$* genes or the *$p16^{INK4a}$* gene alone are described in a wide variety of human tumours, specific deletions of the *$p15^{INK4b}$* gene are found in only a few cases of leukaemia and lymphomas. In contrast, aberrant methylation of the *$p15^{INK4b}$* gene is a frequent event in leukaemia and lymphomas as well as in myelodysplastic syndrome (MDS) (see Tab. 1). Since two groups confirmed independently from each other the prognostic significance of *$p15^{INK4b}$* gene hypermethylation in acute promyelocytic leukaemia (see below), this cell cycle regulator represents the best example for the diagnostic and prognostic potential of epigenetic modifications in hematopoietic neoplasia.

Methylation of the *$p16^{INK4a}$* gene is nearly exclusively found in non-Hodgkin's lymphoma and only very rarely in leukaemia. In lymphoma, *$p16^{INK4a}$* inactivation is clearly associated with high grade and an unfavourable prognosis [149].

The results concerning the aberrant methylation of the *$p15^{INK4b}$* gene in myelodysplastic syndrome are listed separately in Table 3. Several groups observed an increase in the incidence of *$p15^{INK4b}$* gene methylation with increasing blastic involvement of the bone marrow and disease evolution towards AML. But this question is not yet resolved [101]. Uchida et al. [168] demonstrated for the first time that *$p15^{INK4b}$* gene hypermethylation is associated with high-risk MDS, but these authors did not specify their results for the individual MDS subtypes according to FAB or WHO classification. Tessema et al. [161] analysed the epigenetic inactivation of *$p15^{INK4b}$* only in CMML, but with 33 patients their patient cohort is by far the largest for any MDS subtype reported in the literature. They found a hypermethylation of the *$p15^{INK4b}$* gene in up to 58% of patients.

Also for p57 (3%, 2/52 [95]) and for HIC-1 (2/2 [118]) aberrant methylation is described in MDS cases.

*$p21^{CIP1}$*

The *$p21^{CIP1}$* gene is a very strong candidate for participating in tumour progression, because it is not only an important cell cycle regulator [131], like *$p15^{INK4b}$*, *$p16^{INK4A}$* or *$p57^{KIP2}$*, but also a direct target gene of the transcription activating function of the p53 protein, the most frequently altered protein in human tumours [44]. Expression of the p21 protein is induced by wild-type p53 in the presence of DNA damage, leading to apoptosis or cell cycle arrest. p21 is also involved in replicative senescence and terminal differentiation in various cell types.

An important technical point illustrated by the analysis of the promoter of the *$p21^{CIP1}$* gene is the fact that in different malignancies (derived from completely different tissues) different regions might be affected by aberrant methylation [25, 146]. Therefore, great care has to be taken in selecting the region analysed in a given gene. Results published for the same gene but a different malignancy cannot be transferred directly to the study of a different disease or tissue type. For the prognostic significance of *$p21^{CIP1}$* gene methylation see below.

*$p57^{KIP2}$*

The p57 protein belongs to the family of "kinase inhibitor proteins" (KIPs), which also includes $p21^{CIP1}$ and $p27^{KIP1}$. The Cip/Kip family of cyclin-dependent kinase inhibitors (CKIs) is characterized by obligate binding to preformed cyclin/Cdk complexes. In vitro, these CKIs can block the activity of all cyclin/Cdk complexes, albeit with different potencies [131]. The *$p57^{KIP2}$* gene is located at chromosome 11p15.5, a region harbouring several imprinted genes and implicated in sporadic cancers, including those of the breast, liver, and bladder [49]. This region is also linked to the Beckwith-Wiedemann syndrome, which is characterized by the somatic outgrowth of various tissues and by a predisposition to embryonal tumours of these organs [141].

In mice, imprinting of the *$p57^{KIP2}$* gene is strictly regulated by DNA methylation [70], whereas in humans the maternal monoallelic expression of the *$p57^{KIP2}$* gene might be regulated by a mechanism other than DNA methylation.

Frequent aberrant DNA methylation of the *$p57^{KIP2}$* gene was found in lymphoid malignancies of the B-cell phenotype [95], but only rarely in myelodysplastic syndrome and adult T-cell leukaemia, a result which resembles the findings concerning the *DAP kinase* gene (see above). Kikuchi et al. [82] also found in acute myeloid leukaemia a DNA methylation-dependent inactivation in 28% of their cases.

*p73*

The gene coding for the p73 protein is residing on chromosome 1p36.33, a region frequently deleted in neuroblastoma. The gene shows a significant degree of sequence homology to the well-known "Guardian of the Genome" p53 [86], particularly in the central DNA-binding domain and the carboxy-terminal oligomerization domain. These structural similarities are reflected by functional similarities (see [114] and references therein).

The p73 protein can induce cell-cycle arrest, apoptosis, neuronal differentiation and epidermal differentiation. The protein is activated by DNA damage, and this activation can trigger cell-death, independently of the p53 status. The activation of p73 in response to DNA damage seems to involve post-translational modification instead of transcriptional up-regulation. p73 has been shown to regulate the expression of several genes, some of which are also p53-target genes.

For several epithelial cancers loss of heterozygosity at the *p73* locus has been found, whereas in stark contrast to the *p53* gene point mutations are very rare [114]. In acute lymphocytic leukaemia [31, 59] and Burkitt's lymphoma [110] epigenetic inactivation due to promoter hypermethylation is a quite frequent event, whereas the occurrence and frequency of aberrant methylation in non-Hodgkin lymphoma is still under debate (see Table 1).

**Expression of DNA methyltransferases**

Hypermethylation could be explained at least in part by increased expression of DNA methyltransferases in tumour cells. However, concerning the expression of DNA methyltransferases in neoplastic cells conflicting results are reported.

Initially, a marked increase in DNMT mRNA expression was observed [18] which could not be reproduced in subsequent studies by other groups [88] or only to a much lower extent [42, 143]. Due to different methodologies employed and different entities analysed, a direct comparison of the results reported in the literature is difficult.

Studies of the expression of DNMT mRNA in haematological malignancies have been published by three groups (see Table 5). The overall only modest increase in DNMT mRNA expression may be nevertheless of functional importance because a very low constitutive overexpression of DNMT1 (approx. 2-fold) has been shown to be associated with transformation of NIH3T3 fibroblasts [179].

The question of the appropriate transcripts for normalisation is still not solved. Melki and Clark [115], referring to older studies, correctly pointed out that leukaemic blast cells have a reduced proliferation rate in comparison to normal progenitor cells in the bone marrow, but the leukaemic blast cells proliferate nevertheless more than fully differentiated myeloid cells or resting lymphocytes. This means that a high blast count increases the overall proliferation rate of a given sample, which might influence the overall DNMT mRNA expression level measured in this sample. Comparing granulocytes (non-proliferating cells) with lymphocytes (potentially proliferating cells) from healthy donors, Melki and Clark [115] show a clear proliferation dependent up-regulation of DNMT mRNA expression (see Fig. 2 of reference [115]).

Therefore, DNMT mRNA expression data have to be normalised to blast count and proliferation rate which has not yet been done.

**Non-CpG methylation**

For the last decade there has been an ongoing discussion whether cytosine methylation exists in the mammalian genome outside the canonical CpG dinucleotide (see [98] and references therein). In a very interesting study Malone et al. [108] discovered by bisulfite sequencing cytosine methylation in several CC(A/T)GG sites in the human *B29* (Igb/CD79b) gene promoter in primary effusion lymphoma. The authors could show block of transcription factor binding and thereby transcriptional repression by $C^{m}C$(A/T)GG methylation. This non-canonical cytosine methylation seems to be very specific since 22 C(A/T)G and 5 CCG sites were not methylated in the *B29* promoter. Similar methylation events were already described for integrated retroviral sequences [99] and by Southern blot analysis for the myogenic *Myf-3* gene [53].

However, Malone et al. [108] analysed only tumour cell lines whereas Franchina et al. [53] analysed also peripheral lymphocytes for $C^{m}$CTGG methylation in the *Myf-3* gene, but only by Southern blot which is prone to false positive results (see [9] for a discussion of this problem). In a recent study Agirre et al. [4] determined the methylation status of the three CCWGG sites (and an adjacent CpG site) in the promoter of the tumour suppressor gene p53: by combined restriction digest with *Eco*RII (or *Hpa*II) and subsequent amplification with flanking primers the authors found evidence for methylation in 8 of 25 bone marrow samples from acute lymphoblastic leukaemia patients. Since this approach is also prone to false positive results by failure of complete digestion of the target sequence, these interesting results have to be confirmed by an alternative method.

Taken together, definitive experimental evidence for $C^{m}C(A/T)GG$ methylation in endogenous mammalian genes in vivo provided by bisulfite sequencing is still missing.

**Therapeutic Options**

Recalling the definition of epigenetic phenomena cited in the introductory paragraph ("heritable, but potentially reversible") it is obvious that aberrant DNA methylation as a reversible modification of the genome in the course of neoplastic transformation is an attractive target for drug development and subsequent clinical trials. But it is also obvious that in pursuing this approach specificity is the main problem, given the fact, that the establishment and maintenance of correct methylation patterns during development and differentiation is an essential physiological process.

In the following paragraphs some basic facts and promising results concerning the therapeutic use of DNA methylation inhibitors will be summarised.

DNA demethylation can take place passively during cell division because the newly synthesised daughter strands initially are not methylated (and have to be actively methylated in order to maintain the appropriate methylation patterns). Alternatively, DNA demethylation can be achieved actively by excision of the methylated base [84] or by removal of the methyl group from carbon atom no. 5 [19, 24, 140]. The latter possibility is still a matter of controversy [20, 177].

All demethylation strategies described in the literature and tested *in vitro* as well as *in vivo* inhibit by one means or the other the active methylation after DNA replication, thereby leading indirectly to demethylation during subsequent rounds of cell division.

The anti-leukaemic effect of 5-aza-cytidine and 5-aza-2'-deoxycytidine is not only based on the demethylating activity of these compounds but also on direct cytotoxicity in higher concentrations. Therefore, the studies using both aza-nucleotides can roughly be divided into two groups: those using high-dose therapy, mostly as single-agent, and those using lower doses, mostly in combination with other drugs (for further details concerning the different therapy regimens, see [100]).

The use of demethylating agents has been described for the treatment of acute and chronic myelogenous leukaemia as well as for the myelodysplastic syndrome [93, 100, 150, 154]. In a meta-analysis of 200 AML patients treated in different studies with 5-azacytidine, a response rate (defined as complete remission) of 20% was observed. Partial remission was achieved in 16% [170]. With the deoxy-derivative 5-aza-2'deoxycytidine (decitabine) a

response rate of more than 30% was achieved. In general, the response rate was higher when decitabine was combined with conventional therapies (like amsacrine or idarubicin) [175, 176]. In the subgroup of AML patients with normal karyotype, complete remission could be achieved in up to 60%. It should be mentioned that in the vast majority of cases included in this meta-analysis 5-azacytidine was used in combination with other drugs in second-line therapy.

The first salvage therapy of chronic myelogenous leukaemia patients in the blastic phase with decitabine increased the median survival significantly in comparison to conventional intensive chemotherapy (29 weeks versus 21 weeks). This positive effect was confirmed as an independent parameter by multivariate analysis [147].

Also for the treatment of the myelodysplastic syndromes encouraging results are reported: In a group of 99 patients with high-risk myelodysplastic syndromes treated with 5-azacytidine 60% showed a response [155]. The treatment resulted in significantly higher response rates, improved quality of life, reduced risk of leukaemic transformation, and improved survival compared with supportive care.

Decitabine treatment achieved a response in approximately half of the patients with the highest efficiency in high-risk myelodysplastic syndrome patients [174]. In an extension of the initial study the same authors could show that repeated courses of low-dose decitabine induced cytogenetic remissions in a substantial number of elderly MDS patients with pre-existing chromosomal abnormalities. These remissions were associated with improved survival compared with patients in whom the cyogenetically abnormal clone persisted [104]. For detailed compilations of doses and application schemas see [93, 100, 150, 154].

A very promising new approach might be the combination of demethylating agents with inhibitors of chromatin modifying enzymes because on a molecular level DNA methylation and histone modification act synergistically in the repression of gene expression [22]. Two clinical studies combining the histone deacetylase inhibitor sodium phenylbutyrate with the demethylating agent 5-azacytidine are already described in the literature [93]. First preliminary results demonstrate a reduction of $p15^{INK4b}$ gene hypermethylation accompanied by reduced bone marrow blast count and increased myeloid maturation. Also valproic acid, a well-known drug, and several other substances with histone deacetylase inhibiting activity have a promising potential for the combination with demethylating agents in the treatment of cancer [184].

**DNA hypermethylation and prognosis**

*p15*$^{INK4b}$ hypermethylation in acute promyelocytic leukaemia (APL)

Chim et al. [28] provided in a seminal paper convincing experimental evidence that hypermethylation of the *p15*$^{INK4b}$ gene detected by methylation specific PCR (MSP) is a new molecular marker for disease-free survival (DFS) of patients. This result was recently confirmed in a larger and very homogenous cohort of APL patients [160]. Interestingly, *p15*$^{INK4b}$ gene methylation and also reduced mRNA expression correlated only with disease-free survival (DFS) but not with overall survival (OS) [57].

Acute lymphoblastic leukaemia (ALL)

*p21*$^{CIP1/WAF1/SDI1}$

Roman-Gomez et al. [146] analysed a large cohort of ALL patients (n = 124) and found out that hypermethylation was a significant independent prognostic factor for disease-free survival in both adult and childhood ALL. It turned out that this epigenetic event was fully comparable to, or even surpassed in predictive power, the long-established ALL risk factors like age, BCR-ABL expression, or white blood cell count. But independent confirmation of these very promising results is still missing. Shen et al. published a letter [152] raising several technical concerns and taking the results altogether in question. The reply by Roman-Gomez et al. could not resolve all issues raised by Shen et al. Therefore, the question whether the detection of *p21*$^{CIP1/WAF1/SDI1}$ hypermethylation is of prognostic importance in ALL requires further experimentation.

*Calcitonin*

The prognostic significance of methylation of the *calcitonin* gene in ALL was analyzed by Roman et al. [144] using restriction enzyme digest followed by a semi-quantitative PCR assay. In a cohort of 105 adult and childhood leukaemia patients they found in multivariate analysis that the methylation status of the *calcitonin* gene was an independent prognostic factor in predicting disease free survival (DFS). Complete remission rate was similar in both hypermethylated and hypomethylated patients but the former had a higher relapse and mortality rate.

*Estrogen Receptor* (ER) gene hypermethylation in acute myeloid leukaemia (AML)

Li et al. [94] analysed a large group of patients with acute myeloid leukaemia (n = 261) and found in multiple regression analysis a significant better overall survival (OS) in patients

with methylation of the *estrogen receptor* gene as assessed by Southern Blotting of high molecular weight DNA digested with the methylation-sensitive restriction enzyme *Not*I. Methylation of the *ER* gene had no significant impact on complete remission rate after initial induction therapy and decreased with increasing patient age. The authors propose that a lack of *ER* gene methylation at initial diagnosis might select high risk patients for whom alternate treatment forms should be considered early in their management.

Chronic myelogenous leukaemia (CML)

*ABL1*

One of the first reports correlating aberrant methylation and progression of neoplastic transformation identified the Pa promoter of the ABL gene in the Philadelphia chromosome from CML patients as a target for increasing levels of CpG methylation during progression to blast crisis [185]. Subsequent studies from the same group substantiated these findings [9]. However, the prognostic impact of this epigenetic event is still under debate [8, 76].

*$p15^{INK4b}$*

Concerning aberrant methylation of the *$p15^{INK4b}$* gene, the situation is much less clear than in the case of the *ABL1* promoter. Whereas one group found *$p15^{INK4b}$* hypermethylation in 24% of cases (8/34) and an association with progression [129], two groups analysing 21 and 76 cases, respectively, found no methylation at all [72] or only in 5% of cases (4/76) to a very low degree [85]. As is still very often the case in the methylation field, different methodologies have been applied in all three studies. Therefore, the comparison and interpretation of results is difficult.

Since for the *$p15^{INK4b}$* gene marked intra- and interindividual heterogeneity of methylation patterns is well documented [2, 38, 161], different detection methodologies analysing different potential methylation sites may lead to different and even contradictory results.

*Cadherin-13* (*CDH13*)

The *cadherin-13* gene codes for a new member of the cadherin family, transmembrane glycoproteins mediating calcium-dependent cell-cell adhesion [89]. After epigenetic inactivation of this gene was found for several solid tumours [164, 165], Roman-Gomez [145] demonstrated that hypermethylation of the *CDH13* gene occurs early in CML and is a frequent event in this hematological malignancy (55%, 99/179). Moreover, the authors could show a very good correlation between hypermethylation and absence of CDH13

mRNA expression. Absence of CDH13 mRNA expression was more frequently observed among Sokal high-risk patients, and was also independently associated with a shorter median progression-free survival time and poor cytogenetic response to interferon alpha treatment.

## Conclusion and Perspectives

The steadily growing list of genes inactivated by promoter hypermethylation in hematopoietic malignancies provides not only new insights into the molecular basis of the diseases but also a long list of interesting candidate genes for the development of molecular markers which might contribute to the improvement of diagnosis and also prognosis. In addition, the reversibility of the epigenetic modification, which is highly specific for the neoplastic cell clone, offers new opportunities for therapeutic intervention. Several encouraging results from clinical trials are already described in the literature

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# Table1

Compilation of genes hypermethylated in haematological malignancies.

| Gene | Entity | methylated | Reference | Additional References |
|---|---|---|---|---|
| **bcr-abl** | ALL (adult) | 6/80 (8%) | [59] | [9] |
| | CML | 17/21 (81%) | [129] | |
| | CML | 57/93 (61%) | [76] | |
| | CML | 19/39 (63%) | [48] | |
| | CML | 18/28 (64%) | [185] | |
| | MPD* | 7/97 (7%) | [12] | |
| **Calcitonin** | ALL | 47/105 (43%) | [144] | [90, 118] |
| | ALL | 44/47 (94%) | [162] | |
| | ANLL | 21/22 (95%) | [16] | |
| | AML | 92/133 (69%) | [162] | |
| | CML | 18/39 (46%) | [107] | |
| | CML | 18/51 (35%) | [125] | |
| **DAP-K.** | AML (adult) | 19/45 (42%) | [3] | |
| | AML (pedriatic) | 1/26 (4%) | [80] | |
| | B-ALL | 3/20 (15%) | [80] | |
| | MM | 20/26 (77%) | [128] | |
| | B-NHL | 21/25 (84%) | [80] | |
| **E-cadherin** | ALL | 18/33 (53%) | [32] | [116, 118] |
| | AML | 14/44 (32%) | [32] | |
| **ER** | ALL | 25/27 (93%) | [77] | [118] |
| | ALL | 29/80 (36%) | [59] | |
| | AML | 21/23 (91%) | [77] | |
| | AML | 160/261 (61%) | [94] | |
| | AML | 17/36 (47%) | [166] | |
| **GST-π1** | NHL | 1/47 (2%) | [45] | [118] |
| **HIC-1** | AML | 6/61 (10%) | [78] | [117] |
| **MDR1** | ALL | 36/80 (45%) | [59] | [54, 60] |
| | AML | 11/36 (31%) | [166] | |
| **MyoD** | ALL | 31/80 (39%) | [59] | |
| | AML | 22/36 (61%) | [166] | |
| | B-NHL | 71/92 (77%) | [159] | |
| | NK-/T-NHL | 19/31 (61%) | [159] | |
| | HL | 2/24 (8%) | [159] | |
| **O6MGMT** | NHL | 15/61 (25%) | [47] | |
| | B-DLCL | 30/84 (36%) | [46] | |

| | | | | |
|---|---|---|---|---|
| **p14** | ALL | (0/30) 0% | [153] | [122] |
| | CML | 1/76 (1%) | [85] | |
| | B-NHL* | 0/47 (0%) | [15] | |
| **p15** | ALL | 17/34 (50%) | [178] | [38, 39, 57, 111, 118, 127] |
| | ALL | 19/80 (24%) | [59] | |
| | ALL | 16/30 (53%) | [153] | |
| | B-ALL | 17/36 (47%) | [72] | |
| | B-ALL | 23/41 (56%) | [28] | |
| | B-ALL | 12/29 (41%) | [75] | |
| | T-ALL | 13/23 (57%) | [134] | |
| | T-ALL | 11/23 (48%) | [72] | |
| | T-ALL | 24/77 (31%) | [14] | |
| | AML | 46/65 (71%) | [2] | |
| | AML | 29/55 (53%) | [34] | |
| | AML | 27/42 (64%) | [178] | |
| | AML | 27/29 (93%) | [28] | |
| | AML | 11/36 (31%) | [166] | |
| | AML | 71/87 (82%) | [72] | |
| | AML | 28/42 (67%) | [138] | |
| | AML | 15/29 (52%) | [67] | |
| | AMM | 4/32 (13%) | [173] | |
| | APL | 19/26 ( 73%) | [28] | |
| | APL | 20/65 (31%) | [160] | |
| | ATL | 0/36 (0%) | [130] | |
| | CML | 8/34 (24%) | [129] | |
| | CML | 4/76 (5%) | [85] | |
| | B-NHL* | 30/47 (64%) | [15] | |
| | NHL* | 8/73 (11%) | [112] | |
| **p16** | ALL | 2/80 (2%) | [59] | [38, 57, 60, 72, 111, 122, 127, 134, |
| | B-ALL | 2/41 (5%) | [28] | 136, 158, 169, 178, 183] |
| | T-ALL | 2/79 (3%) | [14] | |
| | AML | 7/32 (22%) | [178] | |
| | AML | 0/29 (0%) | [28] | |
| | AML | 6/36 (17%) | [166] | |
| | AML | 5/20 (25%) | [118] | |
| | AML | 11/29 (38%) | [67] | |
| | AMM | 2/32 (6%) | [173] | |
| | APL | 3/26 (12%) | [28] | |
| | ATL | 28/71 (39%) | [130] | |
| | CML | 0/76 (0%) | [85] | |

| | | | | |
|---|---|---|---|---|
| | MM | 41/101 (41%) | [64] | |
| | B-NHL* | 10/47 (21%) | [109] | |
| | B-NHL* | 15/47 (32%) | [15] | |
| | B-NHL* | 20/62 (32) | [148] | |
| | T-NHL* | 3/29 (15%) | [109] | |
| | NHL* | 11/68 (16%) | [123] | |
| **p21** | ALL | 51/124 (41%) | [146] | [122] |
| | ALL | 0/31 (0%) | [152] | |
| | AML | 0/72 (0%) | [146] | |
| | CML | 0/234 (0%) | [146] | |
| **p27** | B-NHL* | 11/33 (33%) | [123] | [122] |
| | NK-/T-NHL* | 4/23 (17%) | [123] | |
| **p57** | ALL | 42/84 (50%) | [153] | |
| | AML | 7/25 (28%) | [82] | |
| | ATL | 1/51 (2%) | [95] | |
| | DLBCL | 39/71 (55%) | [95] | |
| | MM | 4/26 (15%) | [95] | |
| **p73** | ALL | 11/35 (31%) | [31] | |
| | ALL | 17/80 (21%) | [59] | |
| | ALL | 16/30 (53%) | [153] | |
| | AML | 0/28 (0%) | [31] | |
| | Burkitt´s L. | 8/22 (36%) | [110] | |
| | B-NHL* | 5/28 (18%) | [110] | |
| | NHL | 0/27 (0%) | [31] | |
| **THBS1** | ALL | 16/80 (20%) | [59] | |
| | AML | 9/36 (25%) | [166] | |

* = further subclassification can be found in the original reference

The results are shown in detail only for those studies which analyse more than 20 cases per entity. The column "additional references" contains all studies with less than 20 cases.

# Table 2

Compilation of genes for which only one study reports the analysis of more than 20 cases.

| Gene | Entity | methylated | Reference |
|---|---|---|---|
| CACNA1G | AML | 3/36 (8%) | [166] |
| CD10 | ALL | 8/80 (10%) | [59] |
| c-fms | AML | 18/21 (85%) | [51] |
| GPR37 | AML | 17/36 (47%) | [166] |
| hMLH1 | AML | 2/55 (4%) | [151] |
| hMSH2 | AML | 0/55 (0%) | [151] |
| JunB | CML | 33/33 (100%) | [181] |
| MINT1 | AML | 6/36 (17%) | [166] |
| MINT2 | AML | 2/36 (6%) | [166] |
| MLH1 | AML | 0/36 (0%) | [166] |
| PITX2 | AML | 23/36 (64%) | [166] |
| PTC1A | AML | 6/36 (17%) | [166] |
| PTC1B | AML | 4/36 (11%) | [166] |
| SDC4 | AML | 20/36 (56%) | [166] |
| SOCS1 | AML | 53/89 (60%) | [26] |
| THBS2 | ALL | 42/80 (52%) | [59] |
| TP53 | ALL | 8/25 (32%) | [4] |
| WIT-1 | AML | 17/35 (49%) | [137] |
| SHP1 | ATL | 18/20 (90%) | [133] |

For single studies with less than 20 cases, see text.

# Table 3

Hypermethylation in Myelodysplastic Syndrome

| | RA | RARS | RAEB | RAEB-t | sAML | CMML | RCMD | |
|---|---|---|---|---|---|---|---|---|
| **p15** | 1/11 (9%) | 0/2 (0 %) | 1/5 (20%) | 2/3 (67%) | 2/2 (100%) | n.d. | n.d. | [7] |
| | n.d. | n.d. | 2/6 (33%) | n.d. | 5/5 (100%) | 1/1 (100%) | 0/3 (0%) | [11] |
| | 3/5 (60%) | n.d. | 4/5 (80%) | 4/8 (50%) | 2/2 (100%) | 2/3 (67%) | n.d. | [35] |
| | 1/9 (11%) | 0/2 (0%) | 4/10 (40%) | 4/7 (57%) | 3/5 (60%) | 0/1 (0%) | n.d. | [132] |
| | 4/5 (80%) | 1/1 (100%) | 2/2 (100%) | 4/4 (100%) | n.d. | 2/2 (100%) | n.d. | [138] |
| | 0/10 (0%) | 0/2 (0%) | 7/18 (39%) | 4/5 (80%) | 9/9 (100%) | 0/9 (0%) | n.d. | [139] |
| | 0/9 (0%) | 0/2 (0%) | 3/14 (21%) | 4/10 (40%) | 9/12 (75%) | 1/3 (33%) | n.d. | [163] |
| **p16** | 0/8 (0%) | 0/4 (0%) | 0/6 (0%) | 0/2 (0%) | 0/10 (0%) | 0/2 (0%) | n.d. | [168] |
| | 0/5 (0%) | n.d. | 0/5 (0%) | 0/8 (0%) | 0/2 (0%) | 0/3 (0%) | n.d. | [35] |
| **Calc** | 3/3 (100%) | 4/4 (100%) | 14/15 (93%) | 2/3 (67%) | 1/1 (100%) | n.d. | n.d. | [74] |
| | 6/8 (75%) | 1/2 (50%) | 5/8 (63%) | 1/1 (100%) | n.d. | 0/1 (0%) | n.d. | [37] |

# Table 4

Compilation of studies analyzing methylation and expression of the affected gene

| Gene | Entity | Method | n | Correlation with methylation* | Reference |
|---|---|---|---|---|---|
| **p15** | APL | RT-PCR (semiquant) | 32 | + | [160] |
| **p15** | AML | RT-PCR (quant) | 42 | +/- | [138] |
| **p15** | CMML | real-time RT-PCR | 33 | +/- | [161] |
| **p21** | ALL | RT-PCR | 124 | + | [146] |
| **p27** | BCL | IHC | 25 | +/- | [123] |
| **Jun-B** | CML | RT-PCR (quant) + IHC | 32 | + | [181] |
| **MGMT** | B-DLBL | IHC | 26 | + | [46] |

*: + perfect correlation, +/- correlation in the majority of samples

## Table 5

Expression of DNMT's in hematopoietic malignancies

| Cases | Method | Reference | fold increase | |
|---|---|---|---|---|
| 12 AML | comp. RT-PCR | *β-actin* | DNMT1: 4-5 | [119] |
| 33 AML<br>17 CML | comp. RT-PCR | *GapDH*, *PCNA* | DNMT1: 3-5<br>DNMT3A: 4-5<br>DNMT3B: 3-12 | [120] |
| 5 CLL | real-time RT-PCR | *β-actin*<br>DNMT3A: 2-4<br>DNMT3B: 1 | DNMT1: 2-4 | [115] |

## Figures

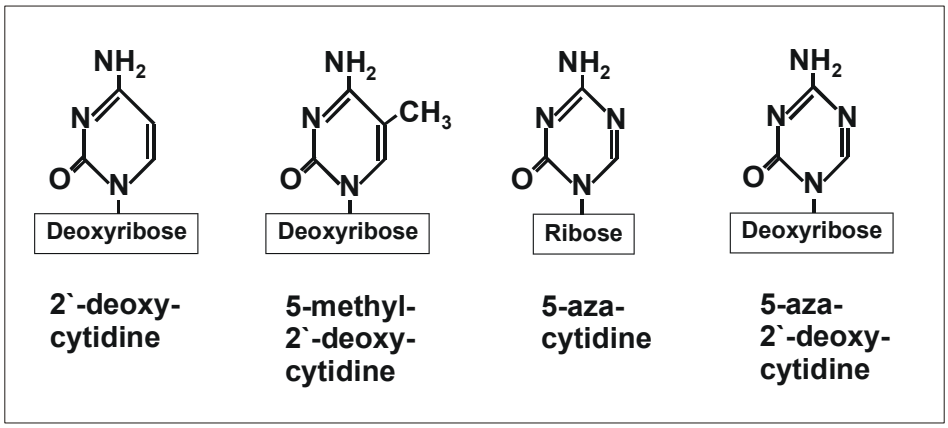



**Fig. 1**

Molecular structure of cytosine, 5'-methyl-cytosine and the demethylating analogues 5-azacytidine and 5-aza-2'-deoxycytidine.

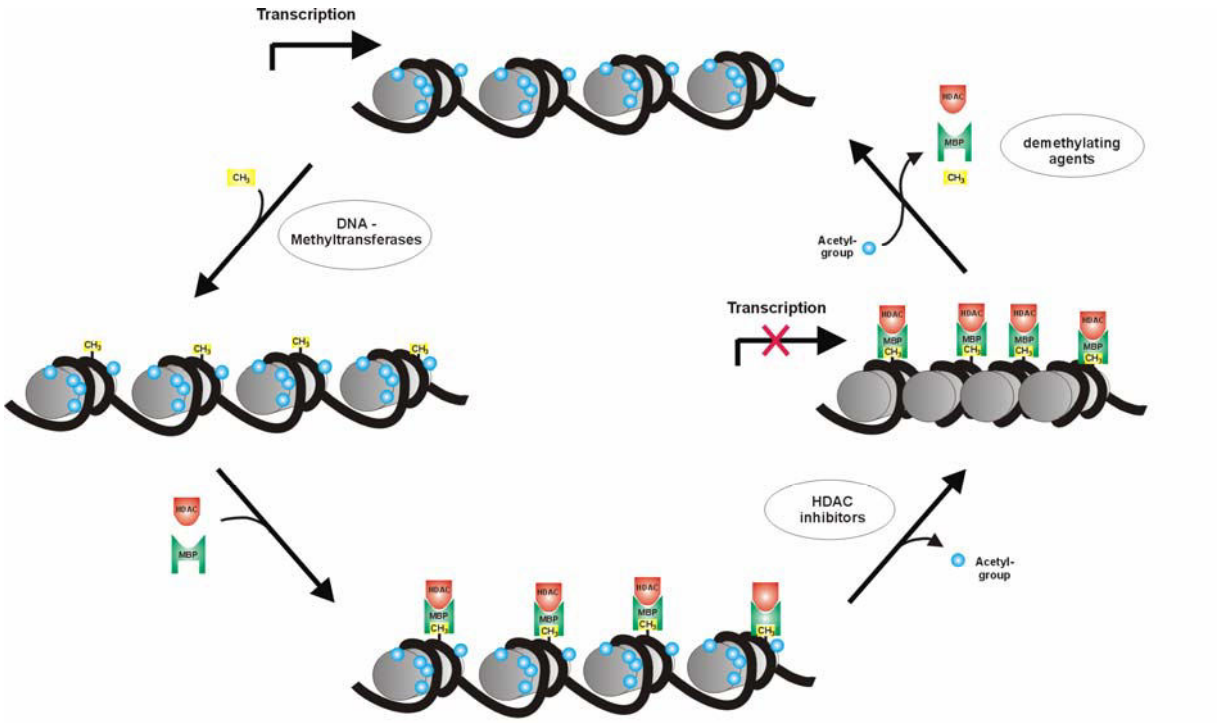
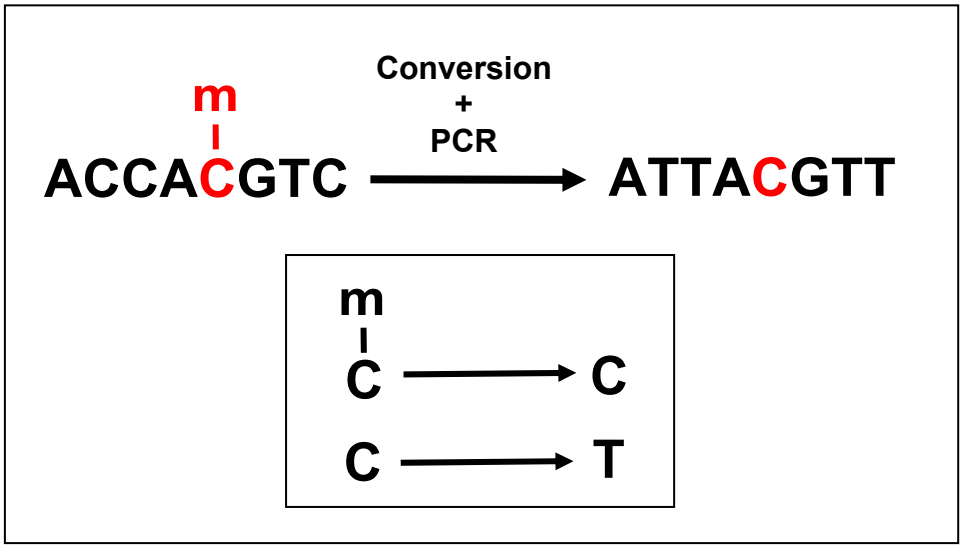



**Fig. 2**

DNA methylation, chromatin structure and gene expression:

Methylated CpG dinucleotides are recognised by methyl-binding proteins (MBP), which in turn recruit histone deacetylating enzymes (HDACs) to the DNA. Histone deacetylation leads to a condensation of chromatin, thereby inhibiting transcription. For a detailed description and further references, see [10].

**A**




**B**

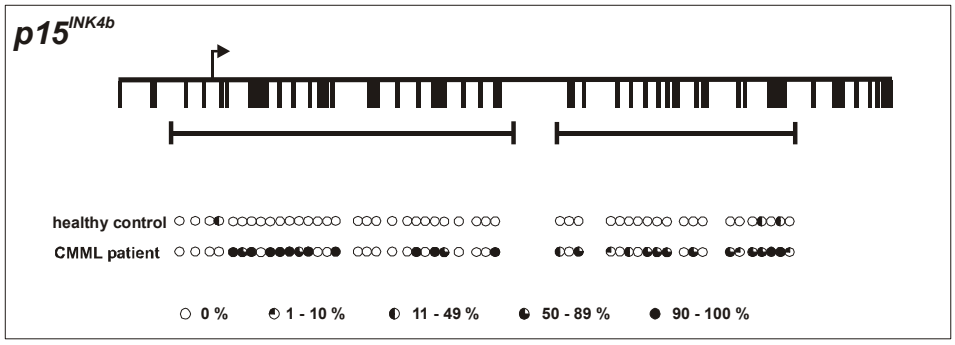



**Fig. 3**

**A)** Principle of bisulfite conversion: cytosine is deaminated in the presence of high concentrations of bisulfite, whereas methylated cytosine remains unaltered. This difference in chemical reactivity translates the epigenetic modification in a difference of the primary sequence.

**B)** Example of the results obtained from direct sequencing of bisulfite treated genomic DNA. The schematic drawing indicates the position of every potential methylation site (CpG dinucleotide) in the promoter and first exon of the *p15*$^{INK4b}$ gene by a vertical bar. In the control biopsy from a healthy individual only occasionally a low level methylation can be found, whereas in the bone marrow biopsy from a CMML patient many CpG sites are nearly completely methylated.

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# Kapitel 2

## Low level of *DAP-kinase* DNA methylation in myelodysplastic syndrome

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**Kai Brakensiek**, Florian Langer, Hans Kreipe, Ulrich Lehmann:
*Low level of DAP-kinase DNA methylation in myelodysplastic syndrome.*
Blood, September 2004, Vol. 104, No. 5, pp. 1586-1588**.** © the American Society of Hematology.
http://www.bloodjournal.org/cgi/content/full/104/5/1586

Recently Voso et al. [1] reported the frequent hypermethylation of the *DAP kinase* gene in myelodysplastic syndrome (in 16 of 34 samples).

Since we are interested in the epigenetic profile of MDS we assessed the methylation status of the *DAP kinase* gene in a larger series of 73 bone marrow biopsies (26 RA, 26 RARS, 21 RAEB) using exactly the same primer set mentioned by Voso et al. (from [2]) and also our published quantitative real-time PCR based methylation assay [3]. In 43% of all MDS biopsies analyzed a signal for methylated DNA could be detected. This frequency is very similar to the one reported by Voso et al. (47%) but the quantitative evaluation of the methylation level in each sample revealed that *DAP kinase* gene hypermethylation is a minor event (methylation level < 5%, see Fig. 1 A). Since low levels of DAP kinase hypermethylation were found in control cases (9/20, methylation level: 0.5-2%) and have also been reported in normal lymphocytes [4], the biological significance of this finding in MDS samples remains unclear.

Employing real-time quantitative RT-PCR we also measured the *DAP kinase* mRNA expression level in 18/20 control biopsies, 12 MDS samples without any methylation and 26/31 MDS samples displaying methylation signals. No significant reduction in *DAP kinase* mRNA level could be observed. On the contrary, we found a weak trend towards increased *DAP kinase* mRNA expression in the MDS biopsies in comparison to the control group (Fig. 1 B), which fits well to the pro-apoptotic function of DAP Kinase [5] and the well known increase in apoptosis in the bone marrow of MDS patients [6]. Parker et al. [7] clearly showed in their study, which is cited by Voso et al. as reference 21, that the rate of apoptosis is significantly increased in CD34+ cells in RA, RARS and RAEB. Therefore this study rather contradicts than supports the statement that "a common feature of MDSs is a decreased apoptosis rate in bone marrow progenitor cells".

Since Voso et al. provide no details of the reaction conditions and do not show any primary data, evaluation of the reported results is difficult. From the context of paragraph one in the "Results and discussion" section we assume that Voso et al., carried out their mRNA expression studies with a subset of AML samples and not with MDS samples, which could explain in part the discrepancy of the results.

The well known differences in the apoptosis rate of RA, RARS and RAEB versus RAEB-t, secondary AML and AML might explain differences in the epigenetic inactivation pattern of the *DAP kinase* gene in these entities which has to be addressed employing quantitative methylation and expression assays. Long term follow-up studies will be necessary to evaluate the significance of low level methylation in MDS for the clonal evolution to AML.

Finally, we would like to mention that already four years ago and also in this journal Aggerholm et al. [8] raised the question of overestimating the proportion of *DAP kinase* gene methylation in AML by using MSP.

## Figure

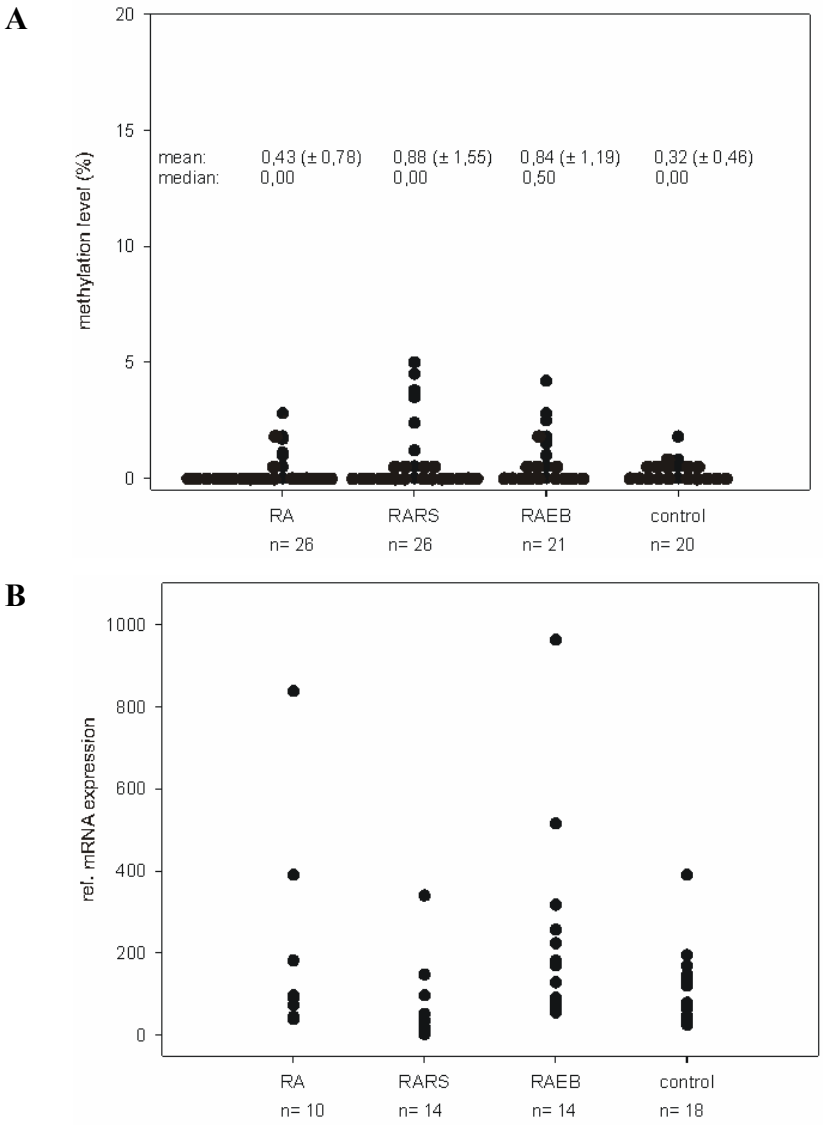


**Fig.1 Methylation and expression analysis of *DAP-Kinase* in MDS.**

**(A)** Results of quantitative methylation analysis of the *DAP kinase* gene in MDS patients and control cases. **(B)** Expression analysis of *DAP kinase* gene in MDS. Measurement of *DAP kinase* mRNA levels using quantitative real-time PCR methodology. The mean expression level of the control group was set to 100% and all individual expression levels were calculated to this mean using the $\Delta\Delta C_T$-method [9]. The mean relative expression level of the MDS samples is 127% ($p = 0.5$, Whiney-Mann-test). Transcript levels were normalized to ß-GUS.

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# Kapitel 3

## Hypermethylation of the suppressor of cytokine signaling-1 (*SOCS-1*) in myelodysplastic syndrome

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**Kai Brakensiek**, Florian Langer, Brigitte Schlegelberger, Hans Kreipe, Ulrich Lehmann: *Hypermethylation of the suppressor of cytokine signalling-1 (SOCS-1) in myelodysplastic syndrome.*
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http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2141.2005.05590.x

## Summary

Transcriptional silencing due to hypermethylation is now recognized to be a hallmark of human tumours. In contrast to acute myeloid leukaemia (AML), comparably little is known about aberrant methylation in myelodysplastic syndrome (MDS), a heterogeneous clonal stem cell disorder with a risk of transformation into secondary AML of up to 30%. Recent evidence demonstrates that *SOCS-1*, a negative regulator cytokine pathways, may act as a tumour suppressor gene, and inactivation due to hypermethylation was shown in various malignancies. Employing a newly developed quantitative real-time PCR based methylation assay we analysed for the first time *SOCS-1* methylation in MDS and found disease-specific hypermethylation in 27/86 MDS patients (31%). Demethylation experiments provided direct evidence that aberrant methylation of *SOCS-1* induces transcriptional silencing in myeloid cells. In addition, by analysing the expression of STAT-induced genes we provide for the first time evidence that the activity of the JAK/STAT pathway is increased in primary patient samples showing *SOCS-1* hypermethylation.

Key words: *SOCS-1*, methylation, MDS, AML, quantitative real-time PCR

## Introduction

Aberration of methylation patterns is a common event in human neoplasia and constitutes a functionally equivalent mechanism to classical genetic alterations like mutations, deletions and allelic losses. Abnormal methylation of cytosine residues in the promoter region is the best studied epigenetic abnormality in tumourigenesis. It was found for several genes and occurs in almost all cancer types, in solid tumours as well as in haematological malignancies (Esteller 2005, Lehmann e*t al,* 2004). A number of studies revealed that hypermethylation of normally unmethylated CpG islands of tumour suppressor genes is associated with transcriptional silencing and thus is assumed to play an important role in cancer development and progression (Herman and Baylin 2003, Jones and Laird 1999). A better understanding of methylation events in tumours will contribute to the improvement of early detection, assessment of prognosis and prediction of therapy response and is therefore also of great practical relevance (Laird 2003, Verma and Srivastava 2002).

Furthermore, aberrant DNA methylation is a reversible modification of the genome and consequently represents an attractive therapeutic target. The most clinically advanced agents, the azanucleosides 5-azacytidine and 5-aza-2'-deoxycytidine (decitabine), provide a very promising approach in haematopoietic malignancies like myelodysplastic syndrome (MDS) and acute myeloid leukaemia (AML) (Ruter e*t al,* 2004). But the data available about aberrant methylation and its role in MDS, a disease characterised by bone marrow dysplasia and ineffective haematopoiesis leading to peripheral refractory cytopenias, are very limited. Apart from studies focussing on $p15^{INK4b}$, little is known about methylation patterns in this disease (for review see Lehmann e*t al,* 2004). Although a progression towards secondary acute myeloid leukaemia (sAML) occurs in up to 30%, depending on the MDS subtype, far more studies on primary AML have been published than on MDS.

One important target for methylation analysis in MDS is the suppressor of cytokine signalling-1 (*SOCS-1*). *SOCS-1* is an important factor in the negative regulation of several cytokine pathways including down-regulation of the JAK/STAT signalling pathway, which is important in the transmission of extracellular cytokine signals to the nucleus and regulates cellular processes involved in cell growth, differentiation and transformation (Alexander 2002, Fujimoto and Naka 2003). The first correlation between *SOCS-1* methylation and cancer was described in hepatocellular carcinoma (HCC) by Yoshikawa Yoshikawa e*t* a*l* (2001). Subsequent studies confirmed these results in HCC (Miyoshi e*t al,* 2004, Okochi e*t al,* 2003, Yang e*t al,* 2003) and found hypermethylation in different other solid tumours (Fujitake e*t* al*,* 2004, Fukushima e*t* al*,* 2003, Lin e*t al,* 2004, Nagai e*t al,* 2003, Oshimo e*t*

*al,* 2004, Sutherland *et al,* 2004, To *et al,* 2004) as well as in haematopoietic diseases (Chen *et al,* 2003, Depil e*t al,* 2003, Ekmekci e*t al,* 2004, Galm e*t al,* 2004, Galm e*t al,* 2003, Liu e*t al,* 2003, Uehara e*t al,* 2003, Watanabe e*t al,* 2004a). Moreover, experimental studies identified tumour suppressor functions of *SOCS-1* (Rottapel *et al,* 2002).

Until now, nothing is known about the methylation of *SOCS-1* in myelodysplastic syndrome. To gain further information about its role in MDS we employed a quantitative real-time PCR based assay and analysed 104 MDS samples. For comparison, 33 AML samples and 24 controls were included in the study.

## Materials and methods

### *Patient samples and cell lines*

Formalin-fixed, paraffin-embedded bone marrow trephines from 86 MDS (24 RA, 26 RARS, 36 RAEB) and 33 AML patients were retrieved from the archive of the Institute of Pathology. In addition, fresh bone marrow aspirates (n = 16) and peripheral blood samples (n = 9) were obtained from 18 patients with clinically and histologically confirmed MDS. Seven corresponding bone marrow trephines (1 without methylation and 6 with methylation in the cell fractions) were available. Mononuclear cells and granulocytes from fresh bone marrow aspirates and peripheral blood samples were isolated performing Ficoll density gradient centrifugation following standard procedures. Patient samples were collected following the guidelines of the local Ethics Committee. Biopsies displaying only non-neoplastic reactive alterations (n = 12) and peripheral blood samples from 12 healthy volunteers, collected after informed consent, were used as control.

### *Cell culture*

Cell lines were purchased from American Type Culture Collection (Rockville, MD) and cultivated according to the supplier's instructions. For the demethylation experiment, KG1a cells were incubated with 3 µM 5-Aza-2'-deoxycytidine for 4 days.

### *DNA isolation and sodium bisulphite conversion*

Genomic DNA isolation from 4-6 10 µm sections using phenol-chloroform after proteinase K incubation and bisulphite treatment were performed essentially as described (Lehmann *et al,* 2001). DNA and RNA from freshly collected cell fractions were isolated using the

"QIAamp DNA Blood Mini Kit" and the "RNeasy Mini Kit", respectively, from Qiagen (Hilden, Germany) following the manufacturer's instruction.

### *RNA isolation and cDNA synthesis*

RNA was extracted exactly as described (Bock *et al,* 2001). Total cDNA was synthesised from 1 µg total RNA using 200 U of SuperScript II RNase$^-$ Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol.

### *Methylation specific PCR (MSP)*

For the qualitative detection of methylated cytosine bases, regardless of the methylation level, conventional MSP was used. The presence of methylated cytosine residues is indicated by an amplification product using the primer pair specific for methylated DNA ("M-primer"). "100%" methylation was defined as the absence of any signal using the primer pair specific for unmethylated DNA ("U-primer") (Fig 1A lanes 9 and 10). The reaction mixture was preheated at 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, 60°C for 45 s, 72°C for 30 s and a final step at 72°C for 5 min. The final volume for each PCR reaction was 25 µl, containing 1x Platinum-Taq reaction buffer, 200 µM of each NTP, 0.625 U Platinum$^{TM}$-Taq (Invitrogen, Karlsruhe, Germany), 400 nM of each Primer, $MgCl_2$ concentration as specified in Table I and up to 3 µl bisulphite-modified DNA. Primer sequences are listed in Table I. PCR products were resolved on 6% polyacrylamide gels.

### *Quantitative methylation specific real-time PCR*

The *SOCS-1* CpG island was examined by real-time PCR amplification (Eads *et al,* 2000) using oligonucleotide primers and a TaqMan probe specific for a fully methylated bisulphite-converted portion of the *SOCS-1* gene. Consequently, only DNA with methylated CpG dinucleotides would be amplified and generate fluorescence signals. As internal reference, *ß-Actin* was used. To convert the measured $\Delta C_T$ values [$C_T$(target) - $C_T$(reference)] into percentage of methylation, DNA-mixtures of completely methylated and completely unmethylated DNA with different concentrations were generated to create a calibration curve (Fig 2).

Since we were able to detect very low levels of methylation in our control samples (mean 0.2% ± 0.3), a stringent threshold was used to exclude false positive results. The mean methylation level in the control group plus 2.58 times the standard deviation (99%

confidence interval) was defined as threshold for scoring a specimen as "hypermethylated". Consequently, we classified all cases ≥1% as methylated.

### *Quantitative real-time RT-PCR*

Quantification of *SOCS-1* mRNA expression was performed by using a hybridisation probe-based real-time RT-PCR. Quantification *of A2M*, *BCL2L1*, *BCL6* and *FAF1* mRNA transcripts was evaluated using SYBR-Green. The housekeeping gene *ß-glucuronidase* (*ß-GUS*) was used as a reference to normalise transcript levels as described (Livak and Schmittgen 2001).

### *Quantitative PCR reactions*

All PCR reactions were performed with an ABI Prism 7700 Sequence Detector (Applied Biosystems). The final volume for each PCR reaction was 25 µl containing 1x Platinum Taq reaction buffer, 1 µM ROX (Invitrogen, Karlsruhe, Germany), 200 µM each of dATP, dCTP, dTTP, dGTP, 0.625 U Platinum$^{TM}$-Taq (Invitrogen, Karlsruhe, Germany), 400 nM of each Primer, 125 nM hybridization probe (or 2µl of a 1:10,000 dilution of SYBR-Green, Molecular Probes, Invitrogen, Karlsruhe, Germany), $MgCl_2$ concentration as specified in Table I and up to 3 µl of bisulphite modified DNA or cDNA, respectively. The reaction mixture containing a hybridization probe was preheated at 95°C for 5 min, followed by 45 cycles at 95°C for 10 sec and 60°C for 1 min. Primer and probe sequences are listed in Table I. For SYBR-Green detection the reaction mixture was preheated at 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s.

### *Statistical analysis*

All analyses were performed using the Statistical Package for the Social Sciences (SPSS) 11.5 for windows. Comparisons between different sample groups were performed with the Chi-Square test. The correlation of methylation levels in the different MDS subtypes was calculated using Pearsons´s correlation test. P-values of $< 0.05$ were considered as statistically significant.

## Results

### *Validation of the SOCS-1 methylation assay*

First, we validated the specificity of our *SOCS-1* assay by testing the primers in methylation specific PCR (MSP) reactions (Herman *et al,* 1996) using bisulfite-modified DNA. GpGenome™ Universal Methylated DNA (Intergen, Edinburgh, UK) was used as positive control and unmethylated lymphocyte DNA as negative control. As expected, amplification of the *SOCS-1* product from Universal Methylated DNA was observed using the M-specific primer set but not with the U-specific primer pair (Fig 1A, lanes 9 and 10). The inverse situation was seen using lymphocyte DNA (Fig 1A, lanes 3 and 4). Furthermore, no PCR product could be amplified using genomic DNA without prior bisulphite treatment (Fig 1A, lanes 5 and 6).

### *Development of a quantitative methylation assay for SOCS-1*

Since qualitative methylation analysis bears the risk of overestimation and therefore misinterpretation of weak signals (even very low methylation levels may result in a clearly visible bands: see Fig 1A, lane 7; Fig 1B), we decided to use a quantitative approach with an additional methylation specific probe (Eads *et al,* 2000). To verify the efficiency of our quantitative *SOCS-1* assay, we performed serial dilutions which demonstrated that the $\Delta C_T$ values [$C_T$(target) - $C_T$(reference)] and thereby the calculated extent of methylation was constant down to the threshold of detection. Four series of DNA-mixtures of methylated and unmethylated DNA with different concentrations were generated to evaluate the sensitivity. Altogether nine experiments were performed and the mean values of them gave a calibration curve (Fig 2) with a very good linear correlation ($r = 0.99$).

### *Hypermethylation of SOCS-1 in different cell lines*

We analysed nine haematological cell lines and found different methylation levels ranging from no methylation at all to 100% methylation (Table II). Only the AML cell line KG1a showed complete methylation.

### *SOCS-1 methylation leads to reduced mRNA expression*

Methylation is known to cause down-regulation or silencing of gene expression. This effect was also shown for *SOCS-1* in several cell lines (Fujitake *et al,* 2004, Fukushima *et al,* 2003, Okochi *et al,* 2003, To *et al,* 2004, Yoshikawa *et al,* 2001) and primary patient

samples (Lin *et al,* 2004, Liu *et al,* 2003, Nagai *et al,* 2003, Okochi *et al,* 2003, Oshimo *et al,* 2004, Sutherland *et al,* 2004, To *et al,* 2004, Yoshida *et al,* 2004). No demethylation experiments were performed so far with the AML cell line KG1a. Therefore, we used KG1a cells as a model to analyse the functional consequences of *SOCS-1* methylation. We treated the cells with 3 µM of the demethylation agents 5-Aza-2'-deoxycitydine and performed in parallel quantitative methylation and expression analysis using real-time PCR. A clear decrease of methylation could be detected whereas the expression level increased (Fig 3). This finding is in concordance with previously described demethylation experiments using other cell lines (Fukushima *et al,* 2003, Galm *et al,* 2003, Liu *et al,* 2003, Reddy *et al,* 2005, To *et al,* 2004, Watanabe *et al,* 2004a) and indicates a good correlation between *SOCS-1* methylation and expression in myeloid cells.

## *SOCS-1 is hypermethylated in MDS and AML*

A mere qualitative analysis of the *SOCS-1* methylation status revealed methylation in 46/86 MDS, 19/33 AML and 20/24 control samples. Employing a threshold (see material and methods for details) none of the 24 control samples was classified as hypermethylated and 19 of the weak MDS signals and 3 of the weak AML signals were excluded. This shows the importance of a quantitative approach, since the qualitative analysis would overestimate and misinterpret background signals. Taken together, we found *SOCS-1* to be methylated in 5/24 (21%) RA, 7/26 (27%) RARS, 15/36 (42%) RAEB and 16/33 (48%) AML samples (Fig 4, Table III). Both, the number of cases found to be methylated and the methylation level correlate with blast-rich MDS subtypes (Pearson´s correlation test: 0.217, $p = 0.018$) (Table III). The statistical analysis revealed significant differences between low risk MDS (RA, RARS), high risk MDS (RAEB) and AML samples compared to the control group ($p = 0.007$, $p < 0.001$ and $p < 0.001$, respectively).

## *SOCS-1 methylation persists in differentiated granulocytes*

To gain further insight into the association of aberrant *SOCS-1* methylation and maturity of haematological cells, we analysed separated cell fractions (mononuclear cells and granulocytes) from 18 MDS patients. Hypermethylation with levels between 1% and 100% was detected in 2/9 peripheral blood samples and 7/16 bone marrow aspirates. All samples with aberrant methylation in the mononuclear cell fraction showed this defect to a similar extent also in terminally differentiated granulocytes (data not shown). The higher and more frequent methylation level in RAEB cases found in the large series of bone marrow

trephines (see above) was also seen as a trend in this series of fresh specimens, but due to the small sample size a statistical analysis was not possible.

### *Impact of SOCS-1 methylation on the expression of target genes of the JAK/STAT-pathway*

To investigate the functional consequences of *SOCS-1* methylation on the activation status of the JAK/STAT signalling pathway, we analysed the mRNA expression level of four STAT-induced genes (*A2M*, *BCL2L1*, *BCL6* and *FAF1*, Dauer *et al,* 2005, Ward *et al,* 2000) in our series of fresh MDS specimens comprising samples with and without *SOCS-1* hypermethylation. The expression of these genes was expected to be elevated, if *SOCS-1* methylation leads to its transcriptional repression and therefore loss of its suppressive function on the JAK/STAT pathway. The expression level of *BCL2L1* was significantly increased in samples harbouring *SOCS-1* hypermethylation (3.4-fold, $p = 0.04$, Mann-Whitney-U-test) (Fig 5). The other three genes showed no statistically significant increase in expression (data not shown).

## Discussion

Here we describe for the first time the analysis of *SOCS-1* methylation in MDS by using, also for the first time, a probe-based quantitative approach. To date limited knowledge exists about methylation in MDS. Most studies focus on the $p15^{INK4b}$ gene and identified $p15^{INK4b}$ methylation as a frequent event (Daskalakis *et al,* 2002, Quesnel *et al,* 1998, Tien *et al,* 2001, Uchida *et al,* 1997). Furthermore, methylation of the *calcitonin* gene was detected (Dhodapkar e*t al,* 1995, Ihalainen e*t al,* 1993), whereas $p16^{INK4a}$ was found to be unmethylated (Daskalakis e*t al,* 2002, Uchida e*t al,* 1997). Aberrant methylation of $p57^{KIP2}$ was described in 2/52 MDS cases and therefore seems to be a minor event (Li e*t al,* 2002). Whether the *DAP-kinase* gene is methylated in MDS is under debate (Brakensiek e*t* al*,* 2004, Voso e*t al,* 2004). So far, nothing is known about the methylation of *SOCS-1*. Therefore this study contributes to a better understanding of the role of methylation in MDS. The region we analysed is located in the *SOCS-1* CpG island and is close to the region in exon 2 which was first described as methylated by Yoshikawa e*t* a*l* (2001) and adopted by nearly all studies analysing *SOCS-1* methylation. The biological and pathophysiological significance of hypermethylation in exon 2 has recently been challenged by one group (Chim e*t al,* 2004a and b, Chim and Kwong 2004). Instead of exon 2 Chim and colleagues

analyzed intron 1, erroneously called “promoter” or “5´-UTR” by these authors (for a correct structure of the gene see: (Watanabe e*t al,* 2004b) and GenBank accession no. Z46940). However, a long list of publications could demonstrate a disease-specific hypermethylation in exon 2 (Chen e*t al,* 2003, Fujitake e*t al,* 2004, Galm e*t al,* 2004, Galm e*t al,* 2003, Guo e*t al,* 2004, To e*t al,* 2004, Watanabe e*t al,* 2004a, Yoshikawa e*t al,* 2001) and recently several studies could convincingly show a strong correlation between exon 2 and transcriptional silencing (Lin e*t al,* 2004, Okochi e*t al,* 2003, Oshimo e*t al,* 2004, Sutherland e*t al,* 2004, To e*t* al*,* 2004, Yoshida e*t al,* 2004).. In line with these recent reports our results also support the suitability of analysing methylation in exon 2: Firstly, we found a disease-specific hypermethylation with different MDS subtypes showing differences in the level of methylation (Table III). Secondly, we showed a clear correlation between exon 2 demethylation and increased *SOCS-1* mRNA expression in KG1a cells (Fig 3). The AML cell line KG1a was used instead of a MDS cell line, since the level of authentication and verification among MDS cell lines is rather low (Drexler 2000).

In contrast to Chim e*t* a*l* (2004a and b), who used a qualitative assay, all our control samples were *SOCS-1* methylation negative. One reason which may explain this is the fact that even very weak methylation levels result in clearly visible qualitative MSP signals (Fig 1A, lane7; Fig 1B).

Several studies have shown a weak background level of methylation signals for several genes (e.g. *DAP-K*, $p15^{INK4b}$, *RASF1A*, *HIN1*, *cyclinD2*, *TWIST,* $p16^{INK4a}$, $O^6$*MGMT*, *GSTP1*, *APC*, *RARß2, CDH1*) in different normal and non-malignant tissues (Brakensiek e*t al,* 2004, Daskalakis e*t al*, 2002, Fackler e*t al,* 2004, Harden e*t al,* 2003, Jeronimo e*t al,* 2004, Toyooka e*t al,* 2002, Trinh e*t al,* 2001). Therefore, these studies clearly demonstrate that a quantitative approach is superior to conventional MSP and it is more and more accepted that a mere qualitative methylation analysis is susceptible to overestimation of weak methylation levels and thus misinterpretation of these low signals (Aggerholm and Hokland 2000, Brakensiek e*t al,* 2004). Quantitative differences, which might be of biological relevance, might remain undiscovered. The best way for a precise methylation analysis is obviously the application of gene-specific thresholds for every tissue or cell type. In the case of *SOCS-1*, a low level of methylation occurs even in normal bone marrow and peripheral blood, but a methylation level higher than 1% is disease-specific.

The results from the small group of patients for which fresh bone marrow aspirates and/or peripheral blood samples were available provide first pieces of evidence for the stable maintenance of aberrant *SOCS-1* methylation during differentiation. Considering the

monoclonal haematopoiesis in MDS one would expect to find complete methylation or no methylation at all in purified blood cell fractions, reflecting the aberrant methylation in the progenitor cell. The heterogeneous levels we found are most probably due to subclonal diversity. But further investigations are needed to clarify this interesting question.

To the best of our knowledge, the observation that the expression of *BCL2L1* is increased in samples harbouring *SOCS-1* hypermethylation represent the first results obtained with primary patient samples that epigenetic inactivation of the *SOCS-1* gene leads, at least partially, to an increase in the activation status of the JAK/STAT-signalling pathway. In future studies these results have to be followed up by a comprehensive analysis of the expression level of all known or putative JAK/STAT target genes in primary patient samples.

**Note added in proof:**

During the revision of this manuscript Johan et al (2005) published in parallel the analysis of *SOCS1* hypermethylation in a MDS and AML series of similar size. Due to different methodology and different primer selection the results are not directly comparably to the results presented in here (Johan et al (2005) employed conventional MSP and the primers are located in intron 1, erroneously called "promoter").

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## Table I. Primer and probe sequences

| Gene | Primer | Sequence (5´ to 3´) | Primer location [a] | mM $MgCl_2$ |
|---|---|---|---|---|
| ***SOCS-1*** | 3M5 | GGA TGG TAG **TCG CG**A GAG TTT **C** | 27779 - 27800 | 3,5 |
| | 3M3 | A**CG CG**A **CG**C TAA **CG**C A**A**C **G** | 27862 - 27880 | |
| | ML-probe | FAM-CCA ACA T**AC G**A**C GCG** A**CG** C**CG**-TAMRA | 27835 - 27855 | |
| | 2U5 | TTT GGA TGG TAG **TTG TG**A GAG TTT **T** | 27776 - 27800 | 1,5 |
| | 2U3 | C**CA** CA**C** **ACA** A**CA** CTA A**CA** CAA **CA** | 27862 - 27884 | |
| | F [b] | TCC TCC GCG ACT ACC TGA | 27964 - 27981 | 2,5 |
| | R [b] | ATG GTT CCA GGC AAG TTA TAA CA | 28055 - 28077 | |
| | RT-probe [b] | FAM-CCT TCC AGA TTT GAC CGG CAG C-TAMRA | 27991 - 28012 | |
| ***β-Actin*** | 1M5 | TGG TGA TGG AGG AGG TTT AGT AAG T | 390 - 414 | 4,5 |
| | 1M3 | AAC CAA TAA AAC CTA CTC CTC CCT TAA | 432 - 461 | |
| | ML-probe | FAM-ACC ACC ACC CAA CAC ACA ATA ACA AAC ACA-TAMRA | 496 - 522 | |
| ***β-Gus*** | F | CTC ATT TGG AAT TTT GCC GAT T | 1779 - 1800 | 2,5 |
| | R | CCG AGT GAA GAT CCC CTT TTT A | 1808 - 1833 | |
| | RT-probe | FAM-TGA ACA GTC ACC GAC GAG AGT GCT GG-TAMRA | 1838 - 1859 | |
| ***A2M*** | F | TTC AGT GGA CAG CTA AAC AGC CA | 911-933 | 1,5 |
| | R | CCT TCT TCT TGG ATC TGG GCC | 1012-1032 | |
| ***BCL2L1*** | F | CCT AAG GCG GAT TTG AAT CTC TTT | 219-242 | 2,5 |
| | R | TAT AAT AGG GAT GGG CTC AAC CAG | 340-363 | |
| ***BCL-6*** | F | TTA AGG CCA GTG AAG CAG AGA TG | 701-723; 794-816 | 1,5 |
| | R | CTC CTC AGT GGC AGG TTG TTC T | 818-839; 911-932 | |
| ***FAF-1*** | F | ATT GGG ACT TAG TGG CAG CTA TCA | 150-173 | 1,5 |
| | R | TAG GAG CTG AAG CTG GAT GAC TTG | 255-278 | |
| ***IRF1*** | F | CAC TCG GAT GCG CAT GAG A | 8-27 | 1,5 |
| | R | TGC TTT GTA TCG GCC TGT GTG | 181-201 | |

Converted cytosines are underlined, CGs are written in bold; [a] nt position in accession number: Z46940 (*SOCS-1*), Y00474 (*β-Act*), NM_000181 (*β-Gus*), NM_000014 (*A2M*), NM_138578; NM_001191 (*BCL2L,* variant 1; variant 2), NM_001706; NM_138931 (*BCL-6,* variant 1; variant 2), AF136173 (*FAF-1*), BT019756 (*IRF1*); [b] Liu et al (2003)

**Table II. SOCS-1 methylation level in cell lines**

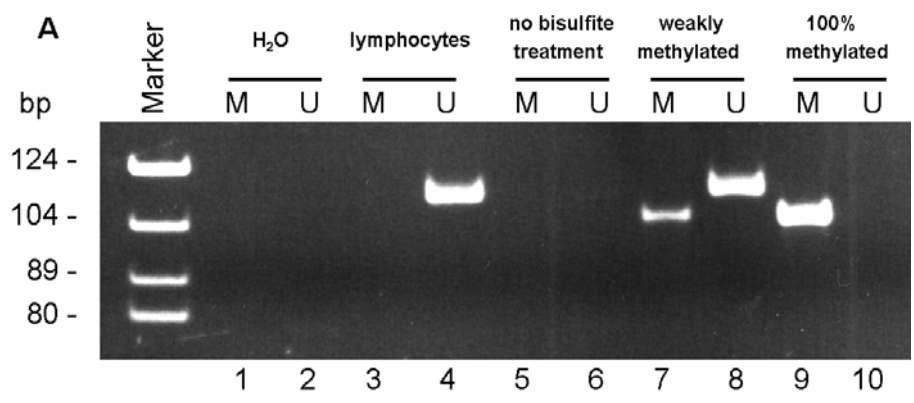
| cell line | origin | methylation level (%) |
|---|---|---|
| KG1a | acute myeloid leukemia | 100 |
| BV173 | chronic myeloid leukemia | 30 |
| RPMI 8226 | multiple myeloma | 0.3 |
| Granta 519 | B-NHL | 0 |
| IM 9 | multiple myeloma | 0 |
| JVM 13 | B-prolymphocytic leukemia | 0 |
| K562 | chronic myeloid leukemia | 0 |
| Raji | Burkitt's lymphoma | 0 |
| Ramos | Burkitt's lymphoma | 0 |

**Table III. SOCS-1 methylation in MDS, AML and control cases**

| | number of patients | | |
|---|---|---|---|
| entity (FAB) | total | methylated [a] (%) | mean |
| RA | 24 | 5 (21) | 2.2 ± 6.2 |
| RARS | 26 | 7 (27) | 3.5 ± 11.8 |
| RAEB | 36 | 15 (42) | 7.6 ± 18.8 |
| AML | 33 | 16 (48) | 11.2 ± 18.9 |
| control | 24 | 0 (0) | 0.2 ± 0.3 |

[a] only cases with a methylation level ≥1% were classified as methylated (see text for detail)

## Figures



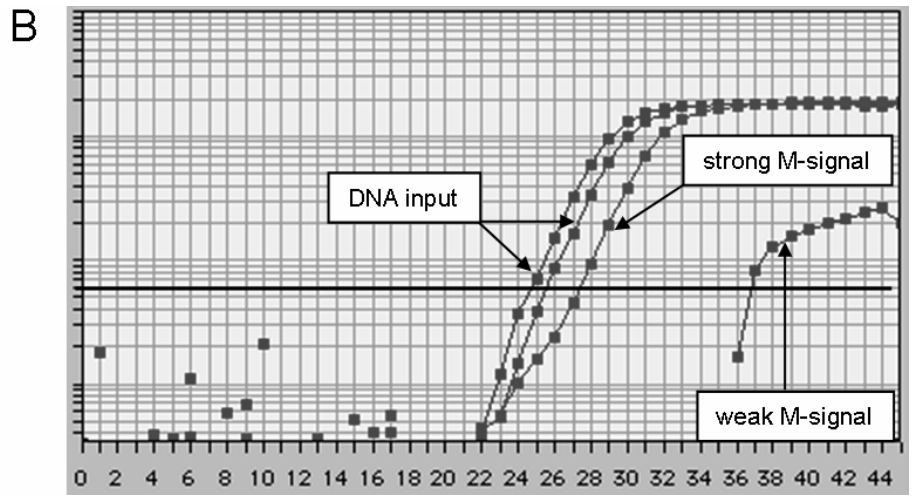




**Fig 1.**

**(A)** Results of a qualitative MSP experiment performed with two primer sets, one specific for methylated DNA (M) and the other for unmethylated DNA (U). No template control (lanes 1 and 2), genomic KG1a DNA (lanes 5 and 6), lymphocyte DNA as negative control (lanes 3 and 4), GpGenome$^{TM}$ Universal Methylated DNA (Intergen) as positive control (lanes 9 and 10) and one sample with very weak methylation level of about 0.2% (lanes 7 and 8) are shown. **(B)** Amplification plot of a quantitative ML-assay. Two samples are shown, one with a very weak methylation level (about 0.2%) and one strong methylation-signal from Universal Methylated DNA (100%). *SOCS-1* signals are evaluated as $\Delta C_T$ values ($C_T$ *SOCS-1* - $C_T$ ß-Actin). The amount of DNA input is illustrated by *ß-Actin*.

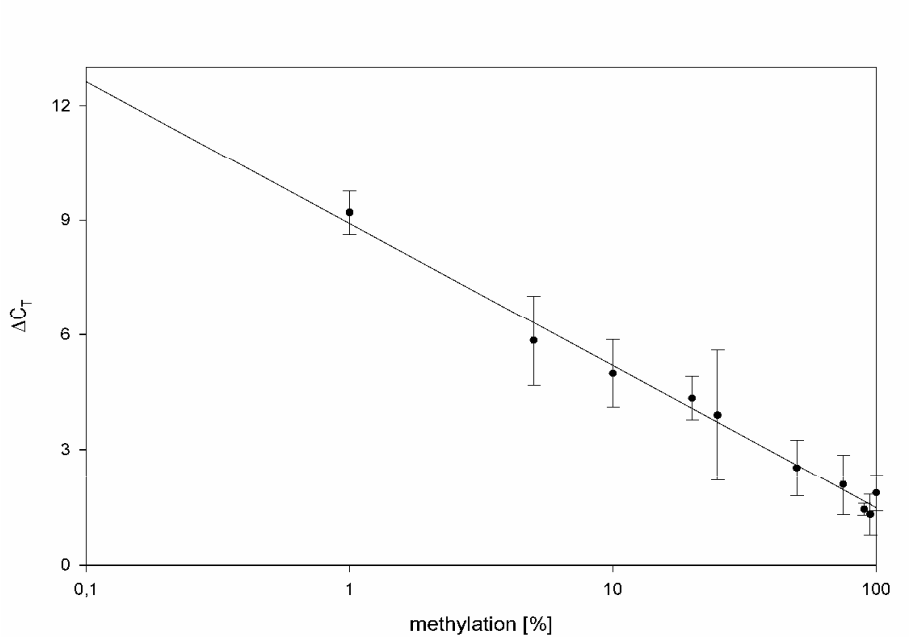



**Fig 2.**

Calibration curve for the quantitative methylation-specific PCR. Mixtures of DNA with a defined methylation status (completely methylated and completely unmethylated) were analysed. Shown is the correlation between extent of methylation and $\Delta C_T$ value (mean of nine independent experiments made with four different series of DNA mixtures).

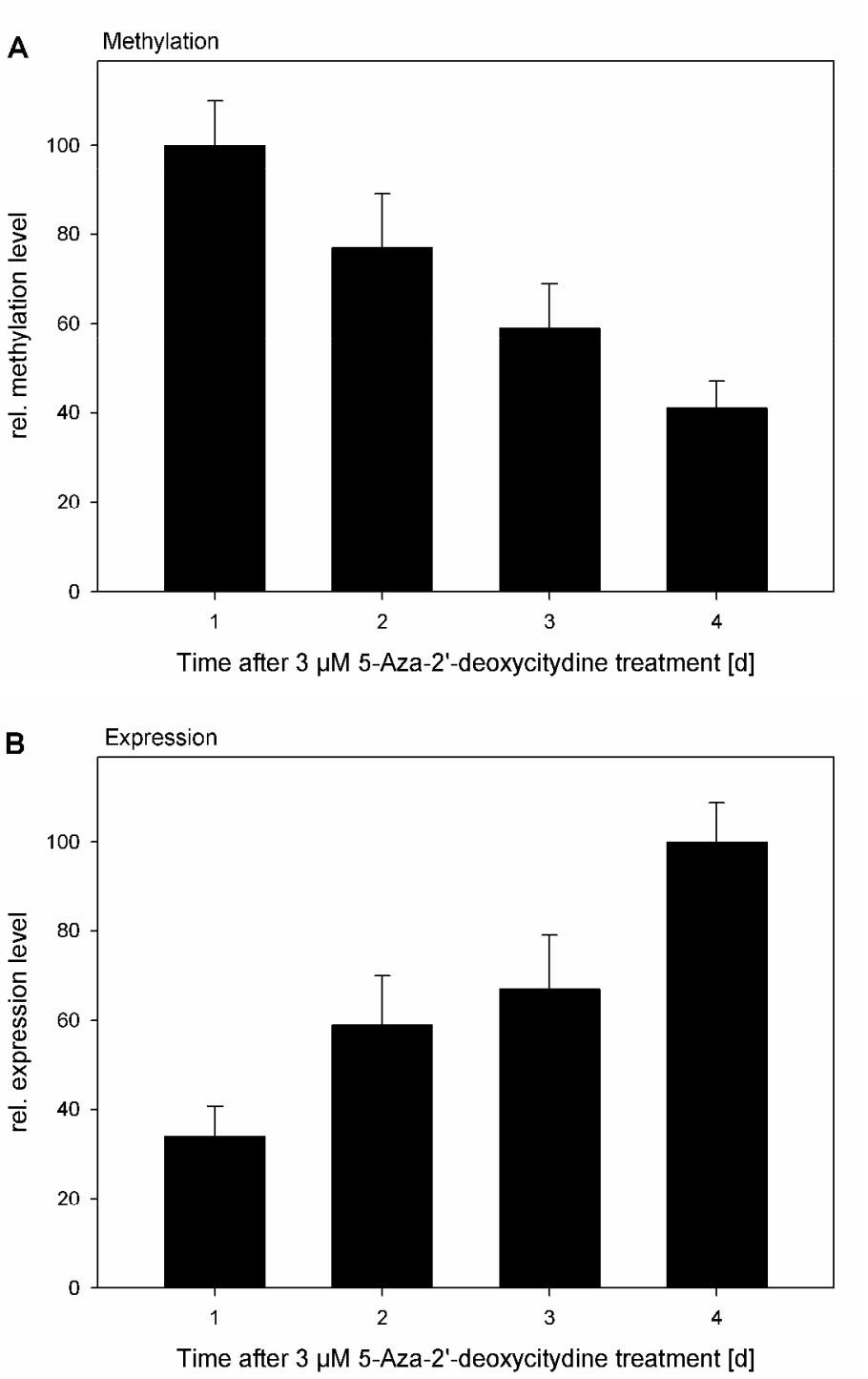



**Fig 3.**

Demethylation studies using 5-Aza-2'-deoxycitydine. KG1a cells were treated with 3 µM 5-azaC for 4 days. **(A)** Methylation and **(B)** expression levels were analysed each day by performing quantitative methylation-specific PCR and quantitative RT-PCR, respectively. Data are presented as mean ± SD calculated from 3 experiments.

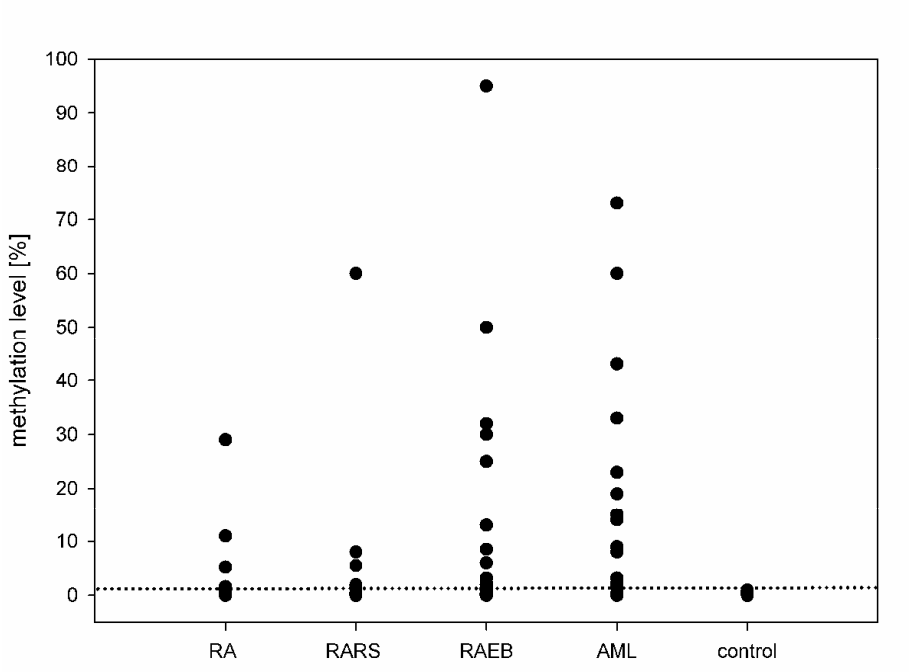



**Fig 4.**

Distribution of *SOCS-1* methylation levels in MDS subtypes, AML and control samples. The threshold was set to 1% (dotted line). All cases exceeding this level were classified as "hypermethylated" (see text for detail).

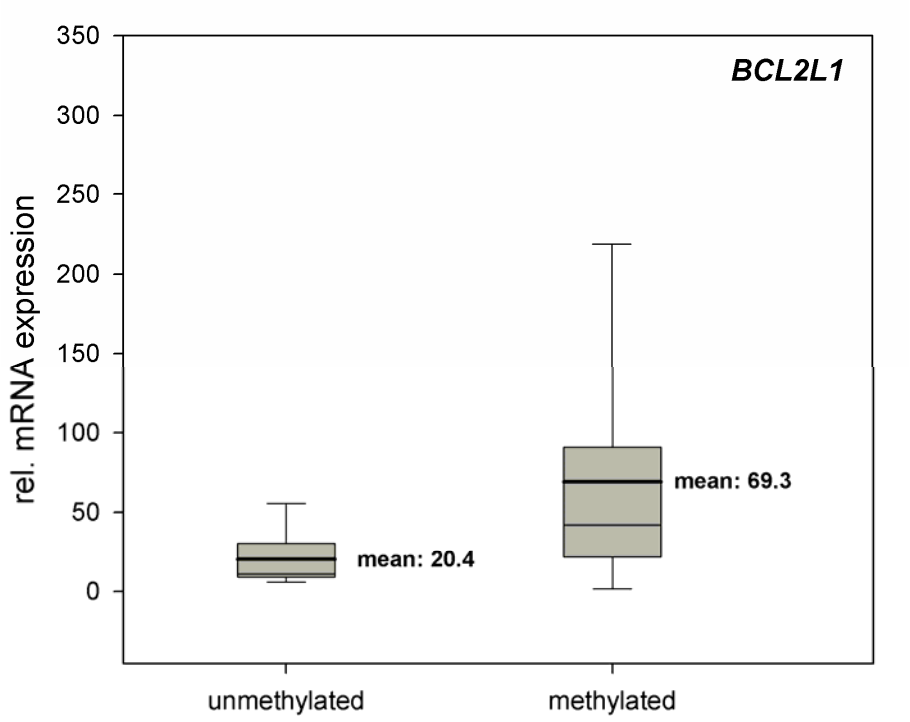



**Fig 5.**

Expression analysis of *BCL2L1*. The expression level of the STAT-induced gene *BCL2L1* was significantly higher (3.4-fold, p = 0.04, Mann-Whitney-U-test) in MDS patient samples with hypermethylation of the *SOCS-1* gene compared to MDS patient samples without any *SOCS-1* gene methylation (Box-and-Whisker-plot). For calculation of the relative expression levels the sample with the lowest expression of the gene under consideration was set equal to one and the expression levels in all other samples were calculated in relation to this sample.

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## Structure and nomenclature of the *SOCS-1* gene

The *SOCS-1* gene consists of two exons separated by an intron of 549 bp in length (see Fig 1). Originally it was thought to be a single exon gene (Naka *et al,* 1997), but later a second exon located 5' to the already known exon was identified (Saito *et al,* 2000, Schluter *et al,* 2000). Interestingly, the mRNA encoded by these two exons contains two open reading frames (upstream ORF "uORF" and major ORF "mORF" = SOCS-1, Accession no. Z46940). It represents therefore an unusual bicistronic mRNA. The functional importance of the first short open reading frame has still to be elucidated.

Most studies analysed hypermethylation of the *SOCS-1* gene, beginning with Yoshikawa *et al* (2001), who reported for the first time aberrant hypermethylation of the *SOCS-1* gene, concentrate on the central part of the CpG islands, which spans the whole gene (Watanabe *et al,* 2004b). Therefore, the primer binding sites are located in the second exon.

Recently, two groups challenged the biological and pathophysiological significance of hypermethylation in exon 2 (Chim *et al,* 2004a, Chim and Kwong 2004, Chim *et al,* 2004b, Johan *et al,* 2005), because analysing the hypermethylation in the intron (see Fig 1: primer pair 1 and 2, respectively) they found discrepancies between exon 2 methylation and methylation in the intron. Unfortunately, both groups call the intron erroneously "promoter" or "5′-UTR". By definition, this region is neither the promoter of the *SOCS-1* gene nor the 5′ untranslated region (UTR) but simply the intron of the *SOCS-1* gene. In this context it has to be stressed that the functional importance of intron methylation is absolutely not clear.

Many groups found disease-specific hypermethylation in exon 2 (Chen *et al,* 2003, Fujitake *et al,* 2004, Galm *et al,* 2004, Galm *et al,* 2003, Guo *et al,* 2004, To *et al,* 2004, Watanabe *et al,* 2004a, Yoshikawa *et al,* 2001) and a correlation between expression and hypermethylation in exon 2 in cell lines (Fujitake *et al,* 2004, Fukushima *et al,* 2003, Okochi *et al,* 2003, To *et al,* 2004, Yoshikawa *et al,* 2001) as well as in primary patient samples (Lin *et al,* 2004, Okochi *et al,* 2003, Oshimo *et al,* 2004, Sutherland *et al,* 2004, To *et al,* 2004, Yoshida *et al,* 2004). Therefore, assessing the methylation status of this region is a well justified approach to identify disease-specific epigenetic alterations which might contribute to an improvement of molecular classification and early diagnosis (Laird 2003).

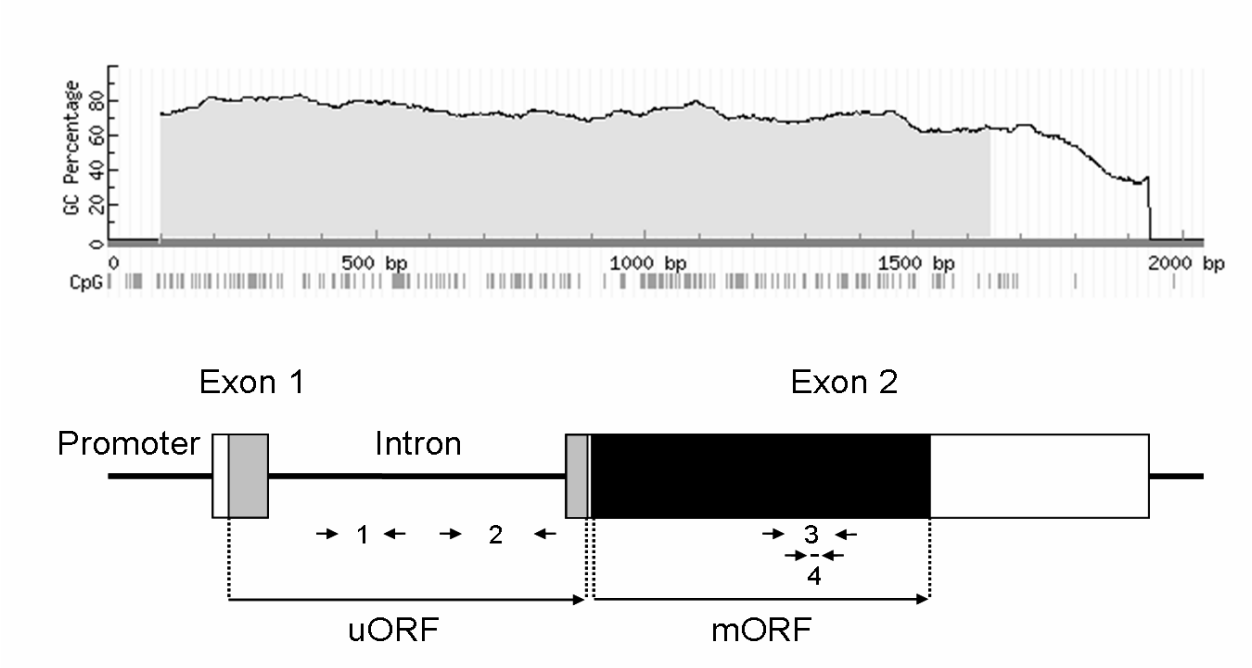


**Fig 1.**

Structure of the *SOCS-1* gene and CpG island. In the upper part, the percentage of the CG count is shown (determined with MethPrimer, from http://www.urogene.org/methprimer). The grey area illustrates the CpG island. Thereunder each CpG site is represented by a vertical line. The lower part shows the structure of the SOCS-1 gene with the two exons, sepatated by the intron. Arrows indicate the different primer positions (1: primer pair used by the Chim group (2004a and b); 2: primer pair used by Johan et al (2005), 3: primer pair from Yoshikawa et al (2001) and 4: our own primer pair and probe (solid line). Abbreviations: uORF, upstream open reading frame; mORF, major open reading frame.

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# Kapitel 4

## Absence of *p21$^{CIP1}$*, *p27$^{KIP1}$* and *p57$^{KIP2}$* methylation in MDS and AML

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**Kai Brakensiek**, Florian Langer, Hans Kreipe, Ulrich Lehmann:
*Absence of p21$^{CIP1}$, p27$^{KIP1}$ and p57$^{KIP2}$ methylation in MDS and AML.*
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## Abstract

Transcriptional silencing of tumour suppressor genes (TSG) due to hypermethylation is a common event in human tumours. The three members of the KIP/CIP family of cyclin dependent kinase inhibitors (CDKIs), $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$, play key roles in cell cycle regulation, but little is known about their methylation in myeloid neoplasia. Therefore, we analysed 9 haematopoietic cell lines, 67 myelodysplastic syndrome (MDS) and 26 acute myeloid leukaemia (AML) cases as well as 11 controls. $p57^{KIP2}$ hypermethylation was found in 4/9 cell lines, but methylation of $p21^{CIP1}$ and $p27^{KIP1}$ was infrequent. All patient samples analysed were methylation-negative for these three genes.

Key words: $p21^{CIP1}$, $p27^{KIP1}$, $p57^{KIP2}$, methylation, MDS, AML, quantitative real-time PCR

## 1. Introduction

Aberrant methylation patterns are a common event in human neoplasia and constitute a functionally equivalent mechanism to classical genetic alterations. It is found for many genes and occurs in almost all cancer types. Especially in hematopoietic neoplasms, DNA methylation abnormalities have emerged as one of the most frequent molecular changes and play critical roles. For example, hypermethylation of the $p15^{INK4b}$ gene, which frequently occurs in myelodysplastic syndrome (MDS), correlates with disease progression [1]. Since aberrant DNA methylation is a reversible modification, it represents an attractive therapeutic target. In clinical studies favourable results were achieved with the usage of methylation inhibitory agents (the azanucleosides 5-azacytidine and 5-aza-2'-deoxycytidine (decitabine)). Treatment of older patients with high-risk MDS and acute myeloid leukaemia (AML) resulted in a significantly higher response rate, improved quality of life, reduced risk of leukaemic transformation and improved survival, compared to best supportive care [2]. Taken together, a better understanding of aberrant hypermethylation of critical target genes which are involved in disease development or progression may positively impact on several clinical issues, including early detection, prognosis and cancer treatment.

Cell cycle progression is regulated by balanced interactions between cyclins and cyclin dependent kinases (CDKs) [3]. The activity of various cyclin/CDK complexes is in turn regulated by a group of regulatory proteins called cyclin dependent kinase inhibitors (CDKIs). Increased expression of CDKIs has been recognized as a general mechanism for cell cycle arrest, and they have also been shown to be implicated in tumourigenesis as tumour suppressor genes (TSG). CDKIs are divided into two families, the INK4 family and the KIP/CIP family.

Abnormalities of the two members of the INK4 family, namely $p15^{INK4b}$ and $p16^{INK4a}$, are intensively investigated. Inactivation by homozygous deletion or alternatively by aberrant promoter hypermethylation is described for a wide variety of solid tumours and haematological malignancies. Especially in myeloid neoplasia the $p15^{INK4b}$ gene is methylated quite often [4]. In contrast to this, much less is known about aberrations of the other family of CDKIs. The KIP/CIP family consists of three members: $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP}$. Mutational inactivation is infrequent and only recently hypermethylation of all three genes has been described in haematological malignancies and was shown to correlate with transcriptional silencing [5-9]. Consequently, gene inactivation by epigenetic mechanisms like promoter hypermethylation may be an important pathway. But until now, only one study analysed methylation of $p57^{KIP2}$ in MDS (2/52 hypermethylated [5]), and two

conflicting studies exist about *p57*$^{KIP2}$ hypermethylation in AML [5, 6]. So far no data exist about the methylation of *p21*$^{CIP1}$ and *p27*$^{KIP1}$ in MDS and AML. For a better understanding of the role of aberrant methylation of the three genes in these malignancies, we examined their methylation status in 9 haematopoietic cell lines, 11 controls and a large series of MDS (n = 67) and AML (n = 26) patients.

## 2. Materials and methods

### *2.1. Patient samples and cell lines*

Formalin-fixed, paraffin-embedded bone marrow trephines from 67 MDS (21 RA, 21 RARS, 25 RAEB) and 26 AML patients and 11 biopsies displaying only non-neoplastic mild reactive alterations were retrieved from the archive of the Institute of Pathology. All patient samples were collected following the guidelines of the local Ethics Committee.

### *2.2. Cell culture*

The cell lines BV173 (chronic myeloid leukaemia), Granta519 (B-NHL), IM9 (multiple myeloma), JVM13 (B-prolymphocytic leukemia), K562 (chronic myeloid leukaemia), KG1a (acute myeloid leukaemia), Raji (Burkitt's lymphoma), Ramos (Burkitt's lymphoma), RPMI8226 (multiple myeloma) were purchased from American Type Culture Collection (Rockville, MD) and cultivated according to the supplier’s instructions.

### *2.3. DNA isolation and sodium bisulphite conversion*

Genomic DNA isolation from 4-6 10 µm sections using phenol-chloroform after proteinase K incubation and bisulphite treatment were performed essentially as described [10]. After bisulphite treatment unmethylated cytosines are converted to uracil (which is amplified as thymidine in subsequent PCR assays), whereas methylated cytosine remains unaltered.

### *2.4. PCR reactions*

The final volume for each PCR reaction was 25 µl, containing 1x Platinum-Taq reaction buffer, 200 µM of each NTP, 0.625 U Platinum$^{TM}$-Taq (Invitrogen, Karlsruhe, Germany), 400 nM of each Primer, $MgCl_2$ concentration as specified in Table 2 and up to 3 µl bisulphite-modified DNA. A hybridization probe (125 nM) and 1 µM ROX (Invitrogen, Karlsruhe, Germany) was added to the quantitative PCR reactions. GpGenome$^{TM}$ Universal

Methylated DNA (Intergen) as positive control and unmethylated lymphocyte DNA as negative control were used in all the experiments. Primer and probe sequences are listed in Table 2.

### *2.5. Methylation specific PCR (MSP)*

The methylation status of all four genes was assessed by using conventional methylation specific PCR. Appropriate primer pairs specific for either methylated or unmethylated bisulphite treated genomic DNA were employed. The presence of methylated cytosine residues is indicated by an amplification product using the primer pair specific for methylated DNA ("M-primer"). The reaction mixture was preheated at 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, 55°C ($p27^{KIP1}$), 60°C ($p21^{CIP1}$ & $p57^{KIP2}$) or 54°C ($p15^{INK4b}$) for 45 s, 72°C for 30 s and a final step at 72°C for 5 min.

### *2.6. Quantitative methylation specific real-time PCR*

CpG island methylation of $p21^{CIP1}$ and $p57^{KIP2}$ was examined by real-time PCR amplification using oligonucleotide primers and a hybridization probe specific for a fully methylated bisulphite-converted portion of the $p21^{CIP1}$ or $p57^{KIP2}$ gene, respectively. As internal reference for DNA input, *ß-Actin* was used. This approach enables the comparison of methylation in different patient samples by quantifying the methylation specific signal relative to the total amount of genomic DNA in the sample. The reaction mixture was preheated at 95°C for 5 min, followed by 45 cycles at 95°C for 10 s and 60°C for 1 min. To evaluate the methylation level, mixtures of methylated and unmethylated DNA with different concentrations were prepared and a calibration curve was generated.

## 3. Results and discussion

The three members of the KIP/CIP family of CDKIs, $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP}$, are important for cell cycle regulation and act as tumour suppressor genes under many circumstances. A decreased expression of them has been documented in a variety of tumours. Inactivating mutations are infrequent, but epigenetic silencing by aberrant methylation has been described [7-9]. Until now, nothing is known about methylation of $p21^{CIP1}$ and $p27^{KIP1}$ in myeloid neoplasia and only two studies analysed $p57^{KIP2}$ hypermethylation in these malignancies with contradictory results. One study reported infrequent methylation in MDS (2/52), but absence of methylation in 14 AML cases [5],

whereas another study reported $p57^{KIP2}$ to be methylated in 7/25 AML cases [6]. Therefore, we analysed the methylation status of the three genes in a large series of MDS and AML cases. First we performed a mere qualitative screening using conventional MSP for all three genes. Analyzing the methylation in nine haematopoietic cell lines, we found methylation signals for $p21^{CIP1}$ in JVM13 (B-prolymphocytic leukemia) and Raji (Burkitt's lymphoma), for $p27^{KIP1}$ in Raji and for $p57^{KIP2}$ in BV173 (chronic myeloid leukaemia), KG1a (acute myeloid leukaemia), Raji and Ramos (Burkitt's lymphoma). Subsequently, we analysed the methylation status in 11 controls, 67 MDS and 26 AML samples. All samples were completely negative for methylation of $p27^{KIP1}$, whereas methylated control DNA showed a strong methylation signal (see Fig. 1A). Weak bands for the M-reactions of $p21^{CIP1}$ and $p57^{KIP2}$ were detected in several samples. To investigate their significance in more detail, we developed quantitative real-time PCR based assays. Methylated control DNA showed strong methylation signals for both genes (see Fig. 1B). Re-evaluation of the methylation signals found in cell lines by applying this more stringent quantitative method revealed that $p21^{CIP1}$ methylation levels were only very low: 1% and 2% for JVM13 and Raji, respectively. The $p57^{KIP2}$ methylation levels for BV173, KG1a, Raji and Ramos were 22%, 25%, 50% and 60%, respectively. In contrast, none of the patient samples and controls displayed methylation of $p57^{KIP2}$. Some of the samples showed $p21^{CIP1}$ methylation, but the measured methylation levels were very weak and near the threshold of detection (< 0.2%). Since all $C_T$-values were greater than 35, conventional MSP using 35 cycles would not have picked up these weak signals. Therefore, we classified them as unmethylated. In contrast to these results, we found methylation signals of the INK4 family member $p15^{INK4b}$ in 40/61 (66%) of these MDS cases analysed by MSP (see Fig. 2). This frequency is in accordance with the data described in the literature. We conclude that unlike the members of the INK4 family, most probably hypermethylation of the genes of the KIP/CIP family of CDKIs does not play a major role in the pathogenesis of MDS and AML, even if this may be the case for other haematological malignancies.

## Acknowledgments

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**Table 1 Patient characteristics**

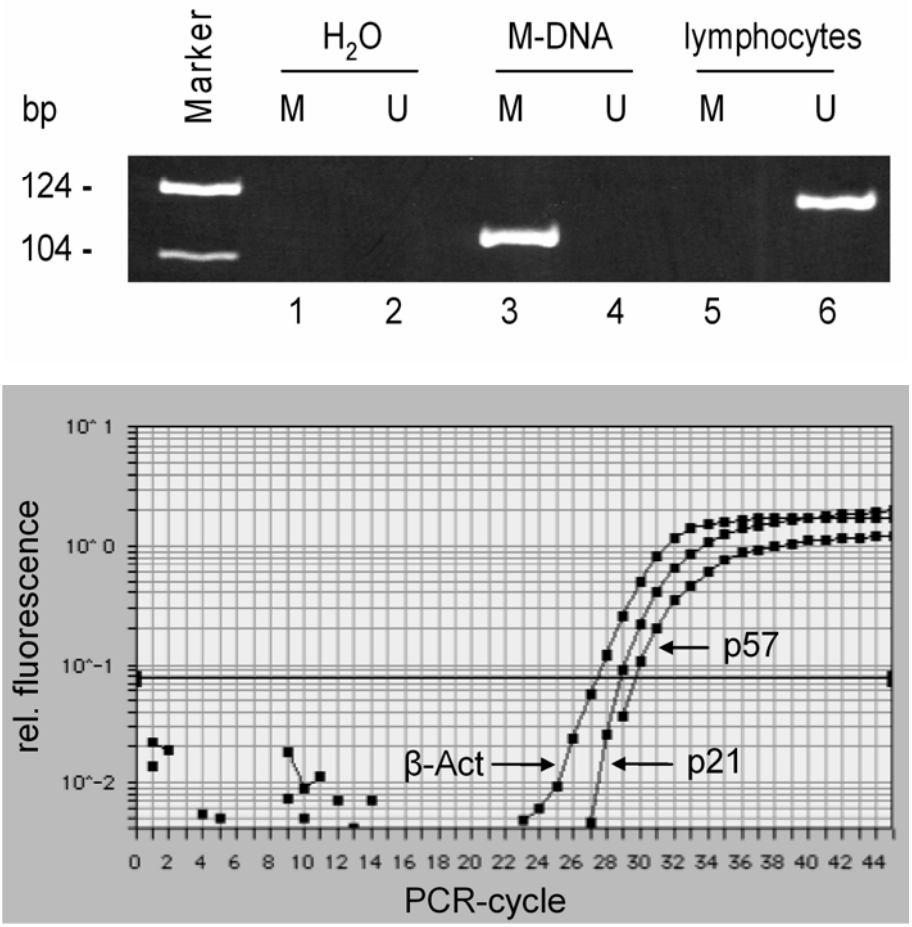
| Diagnosis | No. of patients | Median age (range) | M / F | Erys [$10^6$/µl] | Leucos [$10^3$/µl] | Thrombos [$10^3$/µl] | Hb [g/dl] |
|---|---|---|---|---|---|---|---|
| **RA** | 21 | 75.5 (59 – 91) | 14 / 7 | 2.9 | 4.3 | 158.2 | 8.9 |
| **RARS** | 21 | 76.9 (64 – 90) | 13 / 8 | 4.4 | 5.5 | 264.4 | 8.3 |
| **RAEB** | 25 | 71.3 (57 – 87) | 14 / 11 | 3.2 | 4.2 | 133.9 | 9.1 |
| **AML** | 26 | 67.7 (55 – 81) | 11 / 15 | 4.6 | 39.0 | 89.9 | 8.7 |
| **healthy** | 11 | 63.2 (40 – 83) | 5 / 6 | 4.7 | 7.4 | 164.1 | 14.3 |

Abbreviations: M, male; F, female; Erys, erythrocytes; Leucos, leucocytes; Thrombos, thormbocytes Hb, haemoglobin level

**Table 2 Primer and probe sequences**

| Gene | Primer | Sequence (5´ to 3´) | Product size (bp) | $MgCl_2$ (mM) |
|---|---|---|---|---|
| ***β-Actin*** | 1M5 | TGG TGA TGG AGG AGG TTT AGT AAG T | 133 | 4.5 |
| | 1M3 | AAC CAA TAA AAC CTA CTC CTC CCT TAA | | |
| | probe | FAM-ACC ACC ACC CAA CAC ACA ATA ACA AAC ACA-TAMRA | | |
| ***p21$^{CIP1}$*** | 4M5 | TAC GCG AGG TTT CGG GAT C | 171 | 2.0 |
| | 5M3 | CCC TAA TAT ACA ACC GCC CCG | | |
| | probe | FAM-AAC AAC CCG CGC TCG ACC CA-TAMRA | | |
| | 4U5 | GGA TTG GTT GGT TTG TTG GAA TTT | 161 | 1.5 |
| | 3U3 | ACA ACC CTA ATA TAC AAC CAC CCC A | | |
| ***p27$^{KIP1}$*** | 3M5 | CGA TTA GTT AAT TTT TCG GCG GC | 109 | 1.5 |
| | 3M3 | GCC GAA ACT AAC GAA CGC G | | |
| | 3U5 | AGT TAT GTG ATT AGT TAA TTT TTT GGT GGT | 120 | |
| | 3U3 | CCC CAC CAA AAC TAA CAA ACA CA | | |
| ***p57$^{KIP2}$*** | 2M5 | CGT ATA AAG GGG GCG TAG GC | 138 | 2.5 |
| | 2M3 | CGC CTA TCT CGT CCG AAC G | | |
| | probe | FAM-TTG GGC GTT TTA TAG GTT AAG TGC GTT GT-TAMRA | | |
| | 2U5 | TTT GTG TGT ATA AAG GGG GTG TAG GT | 151 | 1.5 |
| | 2U3 | TCA AAT TCA CCT ATC TCA TCC AAA CA | | |
| ***p15$^{INK4b}$*** | 3M5 | GAG TGT CGT TAA GTT TAC GGT | 118 | 1.5 |
| | 3M3 | AAA CCC TCA TCG CTA CCG | | |
| | 3U5 | GAA GAG TGT TGT TAA GTT TAT | 123 | |
| | 3U3 | CCA AAC CCT CAT CAC TAC CA | | |

## Figures




## Fig. 1.

**Assay validation. (A)** To verify the specificity and sensitivity of our assays, we performed methylation specific PCR for *p27*$^{KIP1}$. In a reaction without template (lanes 1 and 2) we show the absence of unspecific reaction products. Universal Methylated DNA was used as positive control for the "M-reaction" (lanes 3 and 4) and unmethylated lymphocyte DNA was used as control for the "U-reaction" (lanes 5 and 6). **(B)** Quantitative real-time PCR for *p21*$^{CIP1}$ and *p57*$^{KIP2}$ was performed using Universal Methylated DNA as positive control. DNA input is illustrated by *ß-Actin*.

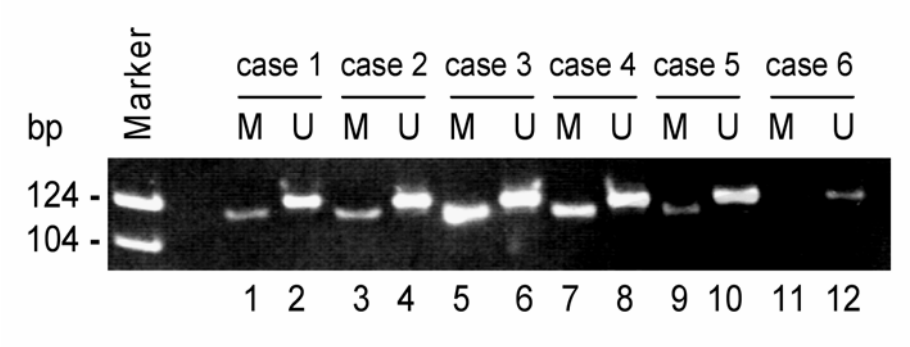



**Fig 2**

Methylation analysis of *p15*$^{INK4b}$ using MSP. Six representative cases from patients with myelodysplastic syndrome are shown. Two cases show strong (case 3 and 4) and three cases show low (case 1, 2 and 5) methylation signals. In one case (case 6) methylation is absent.

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# Kapitel 5

## Quantitative high resolution CpG island mapping using Pyrosequencing™ reveals disease-specific methylation patterns of the *p15$^{INK4b}$* gene in Myelodysplastic Syndrome and Myeloid Leukemia

This research was submitted to Clinical Chemistry.

**Kai Brakensiek**, Luzie U. Wingen, Florian Länger, Hans Kreipe, Ulrich Lehmann: *Quantitative high resolution CpG island mapping using Pyrosequencing™ reveals disease-specific methylation patterns of the p15INK4b gene in Myelodysplastic Syndrome and Myeloid Leukemia.*

## ABSTRACT

**Background:** Gene silencing through aberrant CpG island methylation is the best analyzed epigenetic event in human tumourigenesis with a huge diagnostic and prognostic potential. However, the methylation patterns are often very heterogeneous, which presents a serious challenge for the development of methylation assays for diagnostic purposes.

**Methods:** Employing Pyrosequencing™ technology the methylation status of 68 CpG sites in the CpG island of the $p15^{INK4b}$ gene was determined in a series of bone marrow samples from patients with myelodysplasia and myeloid leukemia (n = 82) and a control group (n = 32). Altogether, 7762 individual methylation sites were quantitatively evaluated. Precision and reproducibility of the quantification was evaluated using several overlapping primers.

**Results:** Using optimized sequencing primers and the new Pyro Q-CpG™ software the quantification of up to 15 CpG sites distributed over approx. 100 bp using a single sequencing primer was precise and reproducible. Extensive statistical analyses of the whole CpG island revealed for the first time disease-specific methylation patterns of the $p15^{INK4b}$ gene in myeloid malignancies and small regions of differential methylation with high discriminatory power, which could discriminate even low grade Myelodysplastic Syndrome (MDS) samples from the control group.

**Conclusion:** Using new Pyrosequencing™ software in combination with optimized sequencing primers the precise quantitative methylation mapping of whole CpG islands is now possible. This reveals disease-specific methylation patterns and enables the development of specific diagnostic assays.


**Key words:** MDS, DNA hypermethylation, $p15^{INK4b}$, Pyrosequencing™

Nonstandard abbreviations: MDS, myelodysplastic syndrome; SNP, single nucleotide polymorphism; RA, Refractory anemia; RARS, Refractory anemia with ringed sideroblasts; RAEB, Refractory anemia with excess blasts; CMML, Chronic myelomonocytic leukemia; AML, Acute myeloid leukemia

## INTRODUCTION

Aberrant DNA methylation of cytosine residues in the promoter region is the best analyzed epigenetic alteration in the development and progression of malignant tumors. It constitutes a functionally equivalent mechanism to classical genetic alterations like mutations, deletions or allelic losses and can be found in almost all cancer types (1). Especially in hematopoietic neoplasms, DNA methylation abnormalities have emerged as one of the most frequent molecular changes and play critical roles (2). Detection of altered methylation has a huge diagnostic and prognostic potential (3), but this requires a comprehensive characterization of the methylation patterns in patient samples and normal tissues to determine which hypermethylation events are disease-specific.

The most commonly used methods are qualitative and quantitative MSP (4), which are very sensitive and easy to use. But both methods can analyze only a very limited number of CpG dinucleotides (approx. 3-10, dependent on the primers/probes used) which are located in the primer/probe binding sites. Furthermore, these methods do not provide precise information about the methylation status of single CpG sites. Bisulphite genomic sequencing is the method of choice for comprehensive methylation mapping, because it provides a single-CpG-resolution over several hundred base pairs. But the detection of small amounts of methylation and the quantification of methylation levels at a given CpG site is very tedious and labor-intensive because it requires the analysis of very large numbers of clones in order to detect and quantify low methylation levels, which is not practical in a diagnostic setting.

Recently, a new real-time DNA sequencing technology, called Pyrosequencing™ was developed for the analysis of single nucleotide polymorphisms (SNPs). This method is based on a sequencing-by-synthesis principle and enables the precise quantification of incorporated nucleotides at polymorphic positions (5, 6). Treatment of the DNA with sodium bisulphite converts the epigenetic difference between methylated and unmethylated cytosine into a SNP of the C/T type (5-methylcytosine remains unaltered, whereas cytosine

is selectively deaminated to uracil and amplified as thymidine in subsequent PCR assays). Therefore, Pyrosequencing™ is a very suitable tool for methylation analysis as well and it is not hampered by the limitations of the techniques mentioned above. It was already shown to be a reliable technique for the precise quantification of methylation at single CpG sites (7-10) with a very good correlation with other methods (11-13). However, the current SNP software provided by the manufacturer is not able to calculate reproducibly (and within a reasonable time frame, i.e. less than a few minutes) all possible sequence combinations, if more than 5 variable positions have to be taken into account.

The tumor suppressor gene *p15*$^{INK4b}$ is well known to be inactivated by hypermethylation in a wide variety of haematological malignancies (2). This methylation is in most cases very heterogeneous and this heterogeneity requires a quantitative analysis with single base pair resolution and provides a methodological challenge for the identification of disease-specific methylation patterns. Improving and validating existing Pyrosequencing™ methodology, we investigated 68 CpG sites using a set of only seven sequencing primers (see Fig. 1), in a large series of patients with myelodysplasia and myeloid leukemia (n = 82) as well as in 32 control subjects. In total, the methylation status of 7762 individual CpG sites was quantitated employing the new Pyro Q-CpG™ software which allows the quantitative analysis of more than 10 CpG sites with a single sequencing primer. A systematic statistical analysis identified characteristic, disease and subtype-specific methylation patterns and revealed two regions of differential methylation with highest discriminatory power.

# MATERIALS AND METHODS

## Patient samples

Formalin-fixed, paraffin-embedded bone marrow trephines from 12 patients with RA, 13 with RARS, 22 with RAEB, 24 with CMML and 11 with AML as well as from 32 controls (biopsies displaying only mild reactive alterations) were retrieved from the archive of the Institute of Pathology. Mean age MDS and AML samples: 76 (median: 78, range: 21 – 91), mean age control group: 55 (median: 58, range: 22 – 86). All samples were collected and processed anonymized following the guidelines of the local Ethics Committee.

## DNA isolation and sodium bisulphite conversion

Genomic DNA was isolated essentially as described (14). Subsequently, DNA samples were treated with sodium bisulphite using the EZ DNA Methylation Kit™ (Zymo Research, HiSS Diagnostics, Freiburg, Germany) following the manufacturer's instruction and finally eluted in 40 µL elution buffer.

## Generation of the PCR-product

PCR products were generated in a 50 µL reaction volume with 400 nmmol/L of forward and reverse PCR primers (see Table 1), 200 µmmol/L of each dNTP, 1.5 mmol/L or 2.5 mmol/L $MgCl_2$ (see Table 1), 1x Platinum-Taq reaction buffer and 1.25 units PlatinumTaq™ (Invitrogen, Karlsruhe, Germany). PCR conditions were 95°C for 5 minutes, followed by 50 cycles with denaturation at 95°C for 30 seconds, annealing at 55°C or 60°C (see Table 1) for 45 seconds, and elongation at 72°C for 30 seconds finished with 1 cycle final elongation at 72°C for 5 minutes. 50 cycles were performed to ensure complete exhaustion of biotinylated primers which could lead to background signals during the Pyrosequencing™ reaction.

## Pyrosequencing™

PCR products (45 – 50 µL) were added to a mix consisting of 3 µL Streptavidin Sepharose HP™ (Amersham Biosciences, Freiburg, Germany) and 37 µL binding buffer (Biotage, Uppsala, Sweden) and mixed at 1200 rpm with a Thermomixer Comfort™ (Eppendorf, Hamburg, Germany) for 5 minutes at room temperature.

Using the Vacuum Prep Tool™ (Biotage, Uppsala, Sweden), single-stranded PCR products were prepared following the manufacturer's instructions. The sepharose beads with the single stranded templates attached were released into a PSQ 96 Plate Low™ (Biotage, Uppsala, Sweden) containing a mix of 45 µL annealing buffer (Biotage, Uppsala, Sweden) and 500 nmmol/L of the corresponding sequencing primer (see Table 1). Pyrosequencing™ reactions were performed in a PSQ 96MA™ System (Biotage, Uppsala, Sweden) according to the manufacturer's instructions using the PyroGold SQA™ Reagent Kit (Biotage, Uppsala, Sweden). CpG site quantification was performed using the new methylation Software Pyro Q-CpG™ (Biotage, Uppsala, Sweden).

Criteria for Pyrogram™ selection were as follows: sufficient peak height of > 15 units (arbitrary units for light emission calculated by the software), symmetric peaks without any irregularities or side-peaks, and a wide reading length with a high reliability till the end of the sequence. Furthermore, the absence of any significant signals at the positions where a bisulfite treatment control was included or where control nucleotides were dispensed to check for unspecific background signals (see Fig. 2).

### Statistical analysis

Statistical analyses were done using the R software package (version 2.1.0). Medium methylation levels per site within each sample group were compared pair wise using the Wilcoxon rank sum test.

For the identification of differentially methylated regions with highest discriminatory power methylation levels were compared over the whole sequence using different window sizes (3 to 8 CpG sites). The mean methylation levels of the 3 to 8 CpG sites within each window were compared among the different sample groups and the resulting p-values were plotted against the start point of the window. All p-values $< 0.05$ were considered as statistically significant.

# RESULTS

## Assay development and validation

Using the PSQ assay design software™ from Biotage as well as a conventional primer design software (Primer Express™, Applied Biosystems), we designed primers to amplify a total of four PCR products with a maximum size of about 300 bp (see Table 1). After polyacrylamid gelelectrophoresis all of these PCR products showed a clear and sharp band without any unspecific side products or primer dimers (data not shown). To analyze every CpG site within these fragments, seven sequencing primers resulting in high quality Pyrograms™ were selected. Figure 2 shows an example (see Materials and Methods for selection criteria).

In order to validate our newly developed Pyrosequencing™ assays with long reading lengths utilizing the new Pyro Q-CpG™ software for multiple methylation sites, we analyzed in a first step placenta DNA, which is known to be unmethylated for most genes, and DNA from the cell line KG1a, which is known to be nearly fully methylated at the $p15^{INK4b}$ CpG island. Accordingly, placental DNA was completely unmethylated whereas KG1a DNA was nearly completely methylated at all 68 CpG sites: mean 94%, range 74 – 100% (mean standard deviation for all CpG sites: 1.1%, data not shown).

Sequencing primers with slightly staggered binding sites gave nearly identical results for every CpG site analyzed, demonstrating that the quantification is sequencing primer independent (compare primer no. 1 and 2 in Fig. 3). Several sequencing primer combinations were positioned in such a way that the last CpG sites reached by one primer (CpG no. 13 – 15 of primer no. 2 in Fig. 3, 60 – 90 bp away from the primer binding site) were the first CpG sites covered by the second primer (CpG no. 1 – 3 of primer no. 4 in Fig. 3). All these measurements gave highly concordant results, demonstrating the reliability of Pyrosequencing™ for the quantitative evaluation of sequences up to 100 bp in length.

## Analysis of patient samples

The quantitative evaluation of up to 15 CpG sites with a single sequencing primer enabled the detailed quantitative mapping of whole CpG islands with a single-CpG-site resolution in an economical way which requires only a few PCR products and sequencing primers. This is a prerequisite for the rational development of diagnostic methylation assays.

To demonstrate the feasibility and power of this approach, we analyzed the very heterogeneous methylation pattern of the CpG islands of the tumor suppressor gene $p15^{INK4b}$ in different MDS subtypes (RA, RARS, RAEB) as well as in CMML and AML samples. In addition, 32 bone marrow samples displaying only mild reactive changes were included in this study as a control group. Altogether 7762 individual CpG sites were analyzed successfully (only 2.5% of potential methylation sites included in the PCR products did not give reproducible results due to technical reasons). The marked intra- and interindividual heterogeneity reported in the literature for the $p15^{INK4}$ gene (e.g. (15, 16)) is clearly confirmed by the quantitative high resolution mapping described in this study. A detailed overview displaying all methylation data for each sample is available as online supplement (Table S 1). Comparing different measurements of the same patient sample the deviation in methylation level at each single CpG site was less than five percentage points. Fig. 4

displays the mean methylation level within each group for every CpG site under investigation. Already the control group shows considerable methylation around CpG no. 25 and beyond CpG no. 50. The mean methylation level of all 68 CpG sites was 5% (± 5%) in the control subjects. In the patient samples mean methylation levels were 6% (± 5%), 6% (± 6%), 11% (± 8%), 11% (± 9%), and 21% (± 13%), for RA, RARS, RAEB, CMML and AML, respectively. Despite the fact that the average methylation levels are quite similar for most subgroups, they increase with disease progression ($r^2$ = 0.79). More important, the distribution of methylated cytosine residues displayed pronounced regional differences (see Fig. 3).

## Identification of small regions with high discriminatory significance

In order to identify the regions showing largest differences in methylation level, the mean methylation levels of all CpG sites within a small window (3 – 8 CpG sites in size) were compared systematically across the whole CpG island between all groups using Wilcoxon rank sum test. The obtained p-values were plotted against the start point of the window. Fig. 5 shows as an example the comparison of the control group with low risk MDS (RA and RARS). Regions displaying statistically highly significant differences are clearly discernible. Even for the comparison of RA and RARS cases, which are sometime difficult to discriminate on clinical and morphological grounds alone, statistical differences were found (p-values < 0.02, data not shown). Only the comparison of RAEB and CMML did not show significant differences in any region. All statistical data are available as online supplement (Table S2 - S7).

## DISCUSSION

Hypermethylation-associated silencing of tumor suppressor genes is the most important and best analyzed epigenetic mechanism in human tumourigenesis. However, all commonly used techniques for the analysis of this aberration (mainly qualitative and quantitative Methylation specific PCR and Bisulfite genomic sequencing) are hampered by one or the other limitation: no quantification of methylation events, a limited number of analysable CpG sites, very labor-intensive or no single-CpG resolution. Pyrosequencing™, a new real-time sequencing technology, possesses the potential to overcome these limitations, which makes it especially useful for the analysis of genes with heterogeneous methylation patterns. Initial reports demonstrated the reliability of this technique for the precise quantification of methylation levels at single CpG sites (7-10) and showed a very good correlation with other methods like COBRA (12), SNaPmeth (11), mass spectrometry (13) and PCR/LDR/Universal array assay (17). The new Pyro Q-CpG™ software (Biotage, Uppsala, Sweden) substantially increases the number of CpG sites that can be analyzed reliably with a single sequencing primer thereby facilitating the analysis of large CpG rich regions (up to whole CpG islands) with only a few sequencing primers. The significantly increased reading length provides also much more flexibility for primer design facilitating the analysis of "difficult" sequences with a very high CpG density. With this advancement we analyzed for the first time up to 15 CpG sites spread over approx. 100 nucleotides with one single sequencing primer. The reliability till the end of the sequencing reaction was confirmed by one or two additional overlapping sequencing primers.

Two alternative tools for the analysis of complex methylation patterns are the base-specific cleavage and subsequent matrix-assisted laser desorption ionisation time-of-flight mass spectrometry of cleavage fragments (MALDI-TOF-MS) (18, 19) and array-based analysis of DNA methylation patterns (20-22). MALDI-TOF-MS is a very promising approach which enables a high-throughput analysis of a large number of CpG sites in many samples. But the

capability of analyzing individual CpG sites in regions with a very high CpG density is often limited due to the absence of suitable cleavage fragments or the generation of identical fragments from different regions. Therefore this method seems to be rather suitable for a high-throughput pre-screening. The success of an array-based methylation analysis is heavily dependent on the composition of the sequence of interest. Even with a comprehensive assay design and the application of very stringent filter criteria for the selection of reliable oligonucleotides, Mund et al. (22) and Kimura et al. (21) were unable to obtain a proper discrimination between individual CpG sites in difficult regions.

A systematic comparison of the methylation patterns revealed region specific differences in the $p15^{INK4b}$ CpG island concerning distribution and level of CpG methylation. The identification of small regions with high discriminatory relevance implies that large areas of the CpG island do not have discriminatory relevance. This demonstrates the necessity for a comprehensive quantitative methylation analysis before high-throughput assays targeting only a few potential methylation sites (e.g. Methylation Specific PCR) can be developed and implemented into the routine diagnostics.

The fact that regions with constitutively methylated CpG sites within the CpG island are already found in the control cases supports the model of "methylation spreading", put forward to explain the hypermethylation of certain regions in cancer cells which are not methylated in normal cells (23, 24). According to this model methylation "spreads" due to loss of inhibitory signals from constitutively methylated areas to adjacent unmethylated sequences.

Occasionally very weak methylation signals were found at positions where control nucleotides were dispensed or where a conversion control was implemented. Consequently, we consider methylation values of up to 5% as potential background signals with questionable significance. This is in concordance with other publications. For example Shaw et al. (10) regard values of 0 – 5% methylation as background "noise" and Jones et al.

(25) defined a threshold of detection of 5% for their JAK2 genotyping assay because they found residual wild type signals of up to 5% in nearly all of their homozygous cases.

In conclusion, we demonstrate that quantitative long read Pyrosequencing™ is a reliable quantitative approach which enables the high resolution mapping of a whole CpG island in a very efficient semi-high-throughput fashion. Employing this technology we could show that only a few quite small regions of the *p15*$^{INK4b}$ CpG island provide relevant information for differential methylation analysis, whereas most regions are inappropriate. Our results show again the advantage of a quantitative compared to a mere qualitative methylation analysis (10, 26, 27) as well as the necessity to analyze the methylation of every individual CpG site. Only this allows a detailed characterization of methylation patterns and identification of relevant regions with high discriminatory power which forms the basis for the development of clinically useful assays. In addition, this will contribute to a better understanding of epigenetic processes at the molecular level.

## Acknowledgments

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## Table 1. Primer sequences, PCR reaction conditions and product sizes

| **forward primer 5´– 3´ (localization)*** | **reverse primer 5´– 3´ (localization)*** | **Temp. $MgCl_2$ Size** | **sequencing primer 5´– 3´ (localization)*** |
|---|---|---|---|
| 4i5:<br>GGGATTAGTGGAGAAGGTG<br>(24792 – 24815) | 1i3:<br>b-AACTCAACTTCATTACCCTCC<br>(25017 – 25037) | 55°C<br>1,5 mM<br>246 bp | pyro 2:<br>GGGGCTAGTGAGGATTT<br>(24824 – 24837)<br>pyro 4:<br>TTTTTTAGAAGTAATTTAGG<br>(24903 – 24922) |
| 2i5:<br>GAGGGTAATGAAGTTGAGTTTAGGTTT<br>(25017 – 25044) | 2i3:<br>b-CCAAAAACTATCRCACCTTCTCCA<br>(25235 – 25258) | 60°C<br>1,5 mM<br>242 bp | pyro 7:<br>TTTTAGGAAGGAGAGAGTG<br>(25044 – 25062)<br>pyro 9:<br>GGTTAAYGGTGGATTATT<br>(25110 – 25126)<br>pyro 22:<br>ATGAGGGTTTGGTTAG<br>(25199 – 25215) |
| 2i5:<br>GAGGGTAATGAAGTTGAGTTTAGGTTT<br>(25017 – 25044) | 10i3:<br>b-CCTAAAACCCCAACTACCTAAATC<br>(25305 – 25328) | 60°C<br>2,5 mM<br>313 bp | pyro 20:<br>GATAGTTTTTGGAAGT<br>(25247 – 25262) |
| 8i5:<br>GGGATTAGTGGAGAAGGTG<br>(25227 – 25245) | 9i3:<br>b-ACCAACRAAAACTCCTATACAAA<br>(25407 – 25429) | 55°C<br>2,5 mM<br>203 bp | pyro 13:<br>TGGGGTTTTAGGGTTT<br>(25317 – 25332) |

* nt position in accession number AC000049; b: biotin

## Figures

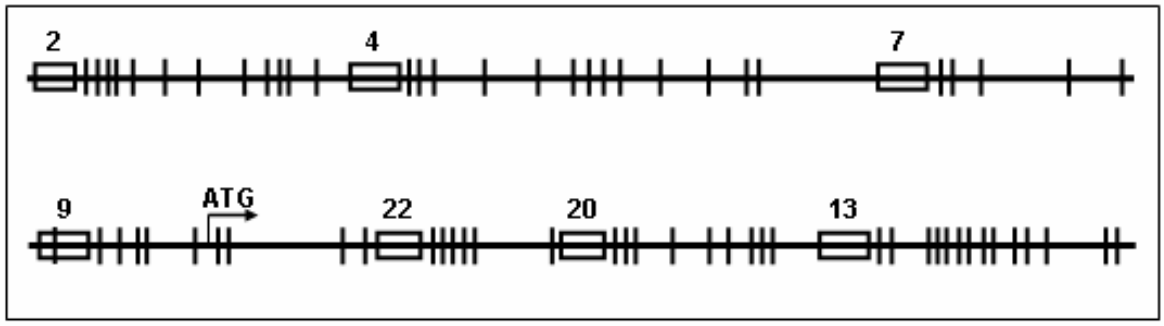


**Figure 1 Location of sequencing primers**

Sequencing primers are represented by rectangles, CpG sites are indicated by vertical bars. Due to assay design reasons CpG no. 46 is located within the binding site of the 3´ PCR primer no. 2 and could not be analyzed.

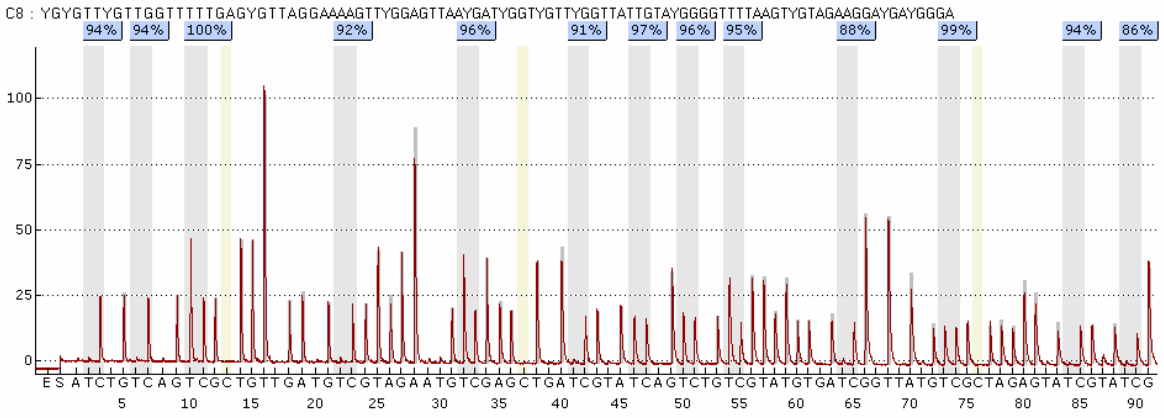


**Figure 2 Representative Pyrogram™.**

Shown is the analysis of the AML-derived cell line KG1a. The sequence in the upper part of each Pyrogram™ presents the sequence under investigation. The grey regions highlight the analyzed C/T sites, with percentage values for the respective cytosine above them. Yellow parts highlight the positions where a cytosine was added to verify the complete conversion from unmethylated cytosine to thymine through the sodium bisulphite treatment.

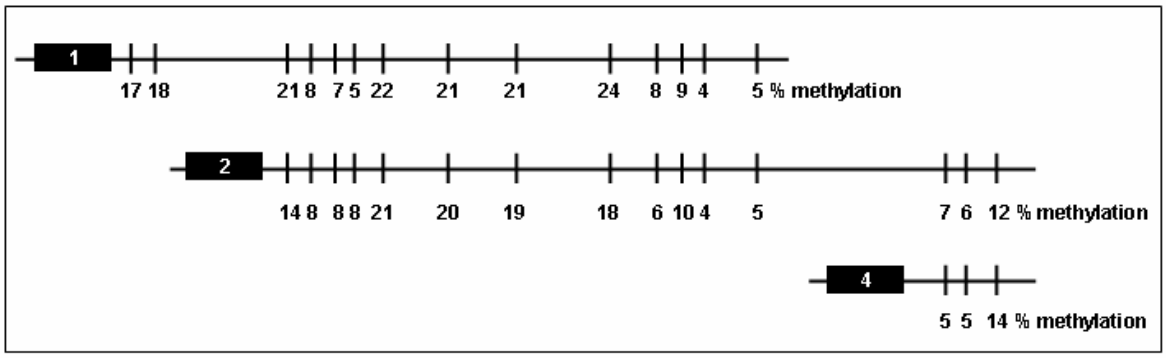


**Figure 3 Demonstration of assay reliability with overlapping primers**

This figure shows exemplarily the results of the analysis with overlapping primers. Sequencing primers are represented by rectangles. CpG sites are indicated by vertical bars with corresponding methylation values below them. The last three CpG sites analyzed with sequencing primer no. 2 have a distance of approx. 90 bp to the primer. Nevertheless, these values are in very good agreement with the values obtained with sequencing primer no. 4.

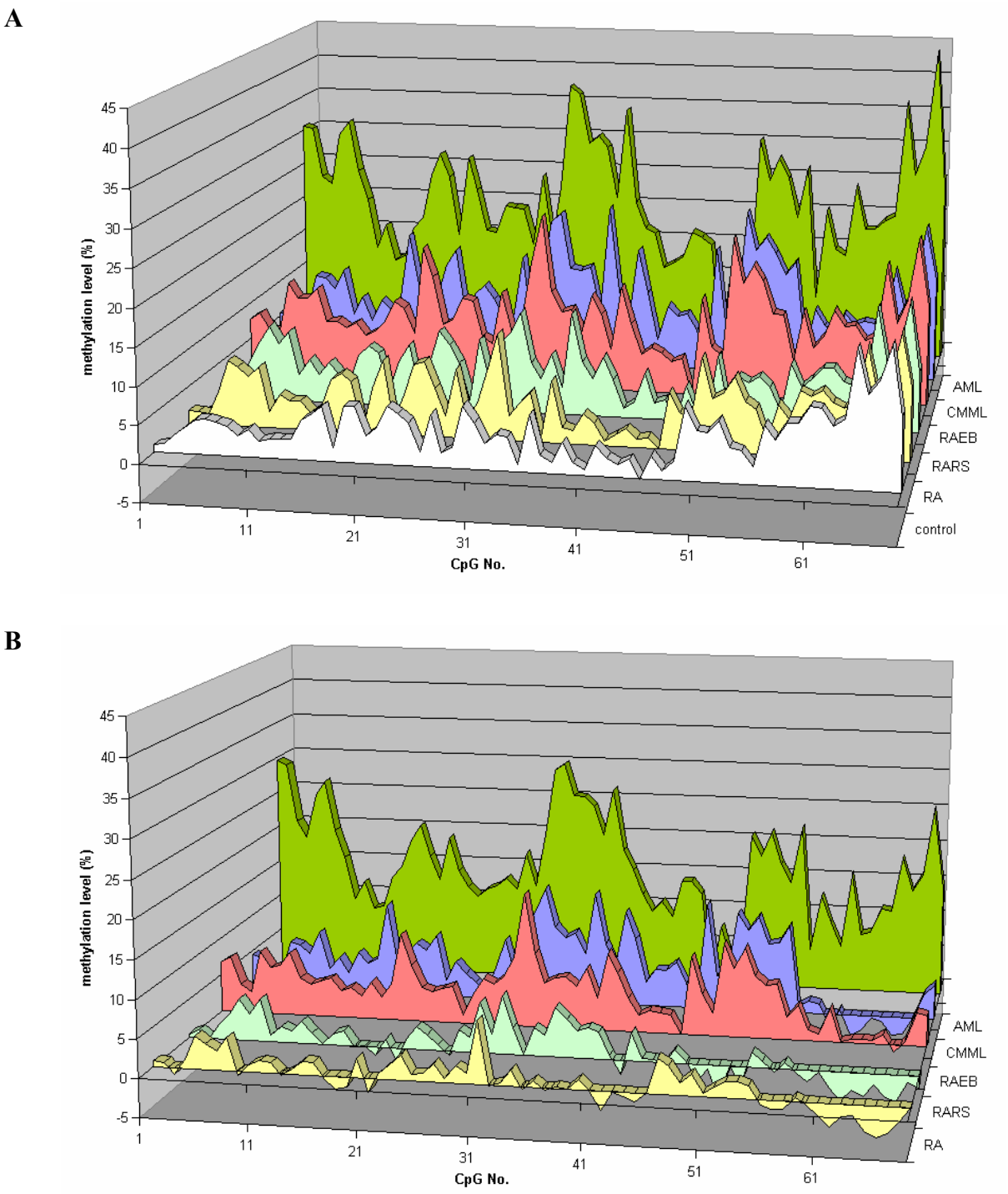


**Figure 4 Quantitative mapping of the *p15*$^{INK4b}$ CpG island**

**(A)** Mean methylation level of every single CpG site in the different groups. **(B)** Relative mean methylation level of every single CpG site (calculated by subtracting the mean value in the control group from the mean value in the patient group for every single CpG site).

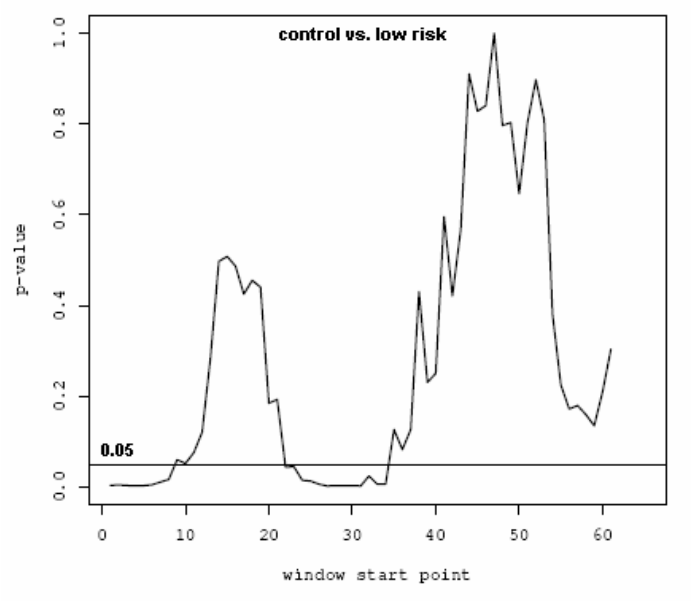


**Figure 5 Detection of differentially methylated regions with discriminatory power**

With a window size of eight nucleotides the whole sequence of all 68 CpG sites was screened for discriminatory relevant regions. The analysis was started at each nucleotide from CpG no. 1 to 61 (x-axis). The corresponding p-values are plotted against the start point of each window. All p-values < 0.05 were considered as statistically significant. In this figure the comparison of controls versus low risk MDS cases is shown as an example. Statistical significant differences were found for the CpG sites 1 – 15 and 22 – 41.

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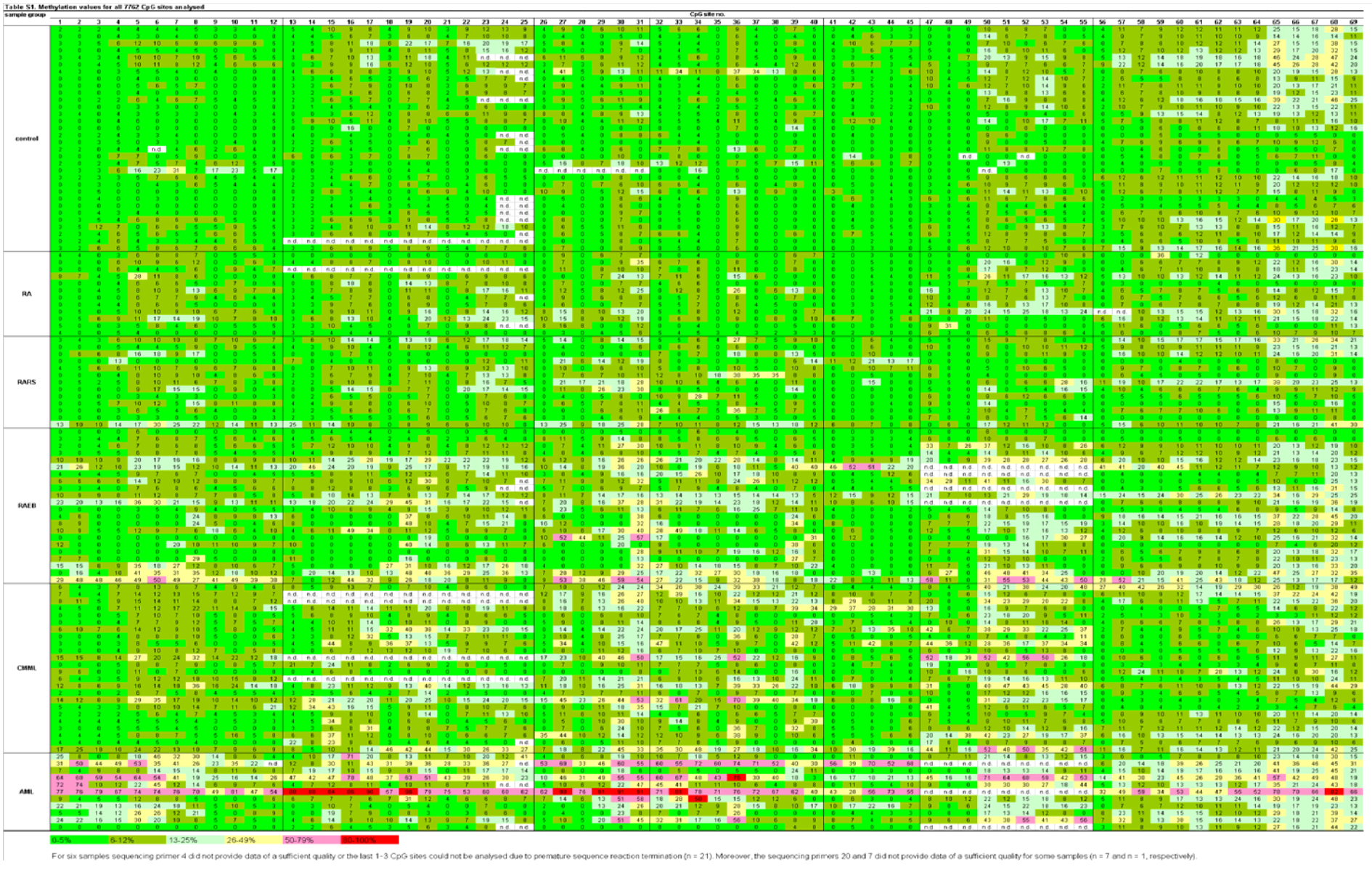
# Anhang zu Kapitel 5

## *Supplementary Material*




For six samples sequencing primer 4 did not provide data of a sufficient quality or the last 1-3 CpG sites could not be analysed due to premature sequence reaction termination (n = 21). Moreover, the sequencing primers 20 and 7 did not provide data of a sufficient quality for some samples (n = 7 and n = 1, respectively).

| Table S2. Statistical results | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| window start point | control | control | control | control | low risk | low risk | low risk | RAEB | RAEB | CMML | RA |
| (window size = 3) | vs. | vs. | vs. | vs. | vs. | vs. | vs. | vs. | vs. | vs. | vs. |
| | low risk | RAEB | CMML | AML | RAEB | CMML | AML | CMML | AML | AML | RARS |
| 1 | 0,941 | 0,000 | 0,004 | 0,000 | 0,001 | 0,013 | 0,000 | 0,337 | 0,006 | 0,001 | 0,887 |
| 2 | 0,497 | 0,032 | 0,028 | 0,000 | 0,158 | 0,149 | 0,000 | 0,716 | 0,008 | 0,002 | 0,785 |
| 3 | 0,047 | 0,198 | 0,001 | 0,000 | 0,724 | 0,203 | 0,001 | 0,817 | 0,016 | 0,003 | 0,913 |
| 4 | 0,001 | 0,148 | 0,000 | 0,000 | 0,781 | 0,336 | 0,001 | 0,384 | 0,010 | 0,003 | 0,806 |
| 5 | 0,001 | 0,023 | 0,000 | 0,000 | 0,940 | 0,322 | 0,001 | 0,415 | 0,010 | 0,003 | 0,765 |
| 6 | 0,001 | 0,000 | 0,000 | 0,000 | 0,423 | 0,262 | 0,003 | 0,809 | 0,021 | 0,012 | 0,605 |
| 7 | 0,007 | 0,000 | 0,000 | 0,000 | 0,043 | 0,101 | 0,007 | 0,523 | 0,157 | 0,060 | 0,414 |
| 8 | 0,018 | 0,001 | 0,000 | 0,001 | 0,057 | 0,080 | 0,019 | 0,817 | 0,400 | 0,188 | 0,462 |
| 9 | 0,141 | 0,003 | 0,005 | 0,004 | 0,063 | 0,115 | 0,040 | 0,741 | 0,503 | 0,285 | 0,661 |
| 10 | 0,021 | 0,002 | 0,001 | 0,002 | 0,196 | 0,125 | 0,130 | 0,826 | 0,553 | 0,631 | 0,495 |
| 11 | 0,020 | 0,000 | 0,000 | 0,003 | 0,005 | 0,013 | 0,061 | 0,956 | 0,878 | 1,000 | 0,623 |
| 12 | 0,092 | 0,000 | 0,000 | 0,003 | 0,003 | 0,000 | 0,037 | 0,545 | 0,789 | 0,845 | 0,956 |
| 13 | 0,179 | 0,020 | 0,000 | 0,000 | 0,094 | 0,000 | 0,000 | 0,076 | 0,056 | 0,577 | 0,450 |
| 14 | 0,028 | 0,199 | 0,000 | 0,000 | 0,473 | 0,000 | 0,002 | 0,133 | 0,077 | 0,457 | 0,663 |
| 15 | 0,067 | 0,380 | 0,000 | 0,001 | 0,683 | 0,000 | 0,009 | 0,055 | 0,050 | 0,869 | 0,862 |
| 16 | 0,529 | 0,212 | 0,000 | 0,004 | 0,280 | 0,001 | 0,013 | 0,256 | 0,125 | 0,725 | 0,601 |
| 17 | 0,628 | 0,002 | 0,003 | 0,003 | 0,001 | 0,002 | 0,002 | 0,762 | 0,492 | 0,397 | 0,705 |
| 18 | 0,610 | 0,000 | 0,002 | 0,006 | 0,001 | 0,012 | 0,011 | 0,537 | 0,774 | 0,495 | 0,908 |
| 19 | 0,885 | 0,001 | 0,007 | 0,016 | 0,001 | 0,009 | 0,023 | 0,472 | 0,939 | 0,522 | 0,862 |
| 20 | 0,696 | 0,002 | 0,004 | 0,029 | 0,015 | 0,027 | 0,057 | 0,659 | 0,702 | 0,522 | 0,931 |
| 21 | 0,564 | 0,010 | 0,064 | 0,017 | 0,041 | 0,207 | 0,052 | 0,391 | 0,541 | 0,420 | 0,601 |
| 22 | 0,114 | 0,015 | 0,103 | 0,010 | 0,202 | 0,953 | 0,140 | 0,332 | 0,504 | 0,148 | 0,862 |
| 23 | 0,135 | 0,038 | 0,597 | 0,010 | 0,301 | 0,352 | 0,081 | 0,290 | 0,201 | 0,043 | 0,931 |
| 24 | 0,076 | 0,002 | 0,025 | 0,011 | 0,085 | 0,548 | 0,135 | 0,516 | 0,491 | 0,278 | 0,935 |
| 25 | 0,156 | 0,006 | 0,010 | 0,022 | 0,098 | 0,123 | 0,229 | 0,930 | 0,422 | 0,670 | 0,723 |
| 26 | 0,244 | 0,012 | 0,003 | 0,058 | 0,149 | 0,072 | 0,336 | 0,708 | 0,985 | 0,972 | 1,000 |
| 27 | 0,023 | 0,025 | 0,000 | 0,016 | 0,528 | 0,027 | 0,229 | 0,366 | 0,454 | 0,957 | 0,662 |
| 28 | 0,045 | 0,019 | 0,000 | 0,000 | 0,249 | 0,002 | 0,004 | 0,428 | 0,104 | 0,213 | 0,354 |
| 29 | 0,007 | 0,000 | 0,000 | 0,000 | 0,032 | 0,003 | 0,009 | 0,475 | 0,117 | 0,214 | 0,765 |
| 30 | 0,005 | 0,000 | 0,000 | 0,000 | 0,004 | 0,001 | 0,003 | 0,886 | 0,097 | 0,114 | 0,446 |
| 31 | 0,003 | 0,000 | 0,000 | 0,001 | 0,002 | 0,001 | 0,036 | 0,982 | 0,169 | 0,227 | 0,495 |
| 32 | 0,019 | 0,000 | 0,000 | 0,002 | 0,007 | 0,000 | 0,011 | 0,435 | 0,067 | 0,177 | 0,806 |
| 33 | 0,019 | 0,006 | 0,000 | 0,000 | 0,044 | 0,000 | 0,004 | 0,560 | 0,060 | 0,160 | 0,661 |
| 34 | 0,032 | 0,006 | 0,000 | 0,001 | 0,130 | 0,000 | 0,007 | 0,222 | 0,078 | 0,271 | 0,210 |
| 35 | 0,060 | 0,009 | 0,000 | 0,000 | 0,072 | 0,000 | 0,006 | 0,391 | 0,174 | 0,346 | 0,096 |
| 36 | 0,083 | 0,018 | 0,000 | 0,000 | 0,171 | 0,007 | 0,013 | 0,403 | 0,193 | 0,355 | 0,300 |
| 37 | 0,039 | 0,000 | 0,000 | 0,001 | 0,001 | 0,002 | 0,028 | 0,912 | 0,804 | 1,000 | 0,036 |
| 38 | 0,029 | 0,000 | 0,000 | 0,002 | 0,001 | 0,002 | 0,046 | 0,271 | 0,985 | 0,817 | 0,081 |
| 39 | 0,028 | 0,000 | 0,000 | 0,005 | 0,001 | 0,000 | 0,065 | 0,523 | 1,000 | 0,606 | 0,112 |
| 40 | 0,605 | 0,002 | 0,001 | 0,001 | 0,004 | 0,002 | 0,001 | 0,502 | 0,207 | 0,434 | 0,476 |
| 41 | 0,691 | 0,194 | 0,013 | 0,007 | 0,064 | 0,008 | 0,003 | 0,326 | 0,092 | 0,286 | 0,489 |
| 42 | 0,512 | 0,430 | 0,017 | 0,011 | 0,212 | 0,008 | 0,004 | 0,233 | 0,112 | 0,364 | 0,475 |
| 43 | 0,993 | 0,312 | 0,012 | 0,040 | 0,449 | 0,021 | 0,054 | 0,302 | 0,183 | 0,617 | 0,348 |
| 44 | 0,293 | 0,014 | 0,000 | 0,024 | 0,083 | 0,000 | 0,091 | 0,176 | 0,591 | 0,709 | 0,806 |
| 45 | 0,049 | 0,000 | 0,000 | 0,014 | 0,018 | 0,001 | 0,095 | 0,620 | 0,674 | 0,859 | 0,478 |
| 46 | 0,233 | 0,000 | 0,000 | 0,036 | 0,013 | 0,001 | 0,197 | 0,741 | 0,717 | 0,472 | 0,080 |
| 47 | 0,717 | 0,000 | 0,000 | 0,000 | 0,002 | 0,001 | 0,002 | 0,839 | 0,522 | 0,408 | 0,036 |
| 48 | 0,253 | 0,000 | 0,000 | 0,000 | 0,001 | 0,000 | 0,000 | 0,990 | 0,192 | 0,223 | 0,072 |
| 49 | 0,584 | 0,000 | 0,000 | 0,000 | 0,000 | 0,000 | 0,000 | 0,829 | 0,210 | 0,133 | 0,191 |
| 50 | 0,398 | 0,000 | 0,001 | 0,000 | 0,000 | 0,000 | 0,000 | 0,919 | 0,090 | 0,151 | 0,076 |
| 51 | 0,693 | 0,001 | 0,000 | 0,000 | 0,006 | 0,002 | 0,000 | 0,839 | 0,156 | 0,191 | 0,495 |
| 52 | 0,681 | 0,012 | 0,001 | 0,000 | 0,056 | 0,003 | 0,001 | 0,542 | 0,126 | 0,133 | 0,313 |
| 53 | 0,904 | 0,239 | 0,001 | 0,000 | 0,432 | 0,003 | 0,002 | 0,205 | 0,077 | 0,182 | 0,382 |
| 54 | 0,815 | 0,438 | 0,025 | 0,000 | 0,543 | 0,034 | 0,000 | 0,422 | 0,022 | 0,012 | 1,000 |
| 55 | 0,699 | 0,751 | 0,954 | 0,003 | 0,454 | 0,617 | 0,003 | 0,826 | 0,041 | 0,007 | 0,827 |
| 56 | 0,584 | 0,785 | 0,568 | 0,002 | 0,515 | 0,912 | 0,004 | 0,582 | 0,043 | 0,004 | 0,978 |
| 57 | 0,535 | 0,846 | 0,451 | 0,002 | 0,448 | 0,849 | 0,002 | 0,538 | 0,034 | 0,002 | 0,785 |
| 58 | 0,398 | 0,979 | 0,174 | 0,002 | 0,455 | 0,704 | 0,001 | 0,385 | 0,025 | 0,001 | 0,605 |
| 59 | 0,132 | 0,986 | 0,179 | 0,001 | 0,189 | 0,960 | 0,000 | 0,252 | 0,029 | 0,001 | 0,624 |
| 60 | 0,067 | 0,778 | 0,147 | 0,002 | 0,220 | 0,826 | 0,000 | 0,355 | 0,012 | 0,001 | 0,549 |
| 61 | 0,057 | 0,958 | 0,236 | 0,001 | 0,140 | 0,541 | 0,000 | 0,355 | 0,007 | 0,001 | 0,460 |
| 62 | 0,285 | 0,666 | 0,529 | 0,000 | 0,216 | 0,542 | 0,000 | 0,385 | 0,002 | 0,000 | 0,828 |
| 63 | 0,231 | 0,798 | 0,403 | 0,000 | 0,212 | 0,562 | 0,000 | 0,422 | 0,001 | 0,000 | 0,957 |
| 64 | 0,243 | 0,840 | 0,545 | 0,000 | 0,224 | 0,418 | 0,000 | 0,552 | 0,001 | 0,000 | 0,828 |
| 65 | 0,247 | 0,454 | 0,895 | 0,000 | 0,114 | 0,230 | 0,000 | 0,468 | 0,000 | 0,000 | 0,913 |
| 66 | 0,421 | 0,115 | 0,278 | 0,000 | 0,056 | 0,112 | 0,000 | 0,613 | 0,000 | 0,000 | 0,765 |

Statistically significant p-values (< 0.05) are highlighted in yellow

| **Table S3. Statistical results** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| window start point | control | control | control | control | low risk | low risk | low risk | RAEB | RAEB | CMML | RA |
| (window size = 4) | vs. | vs. | vs. | vs. | vs. | vs. | vs. | vs. | vs. | vs. | vs. |
| | low risk | RAEB | CMML | AML | RAEB | CMML | AML | CMML | AML | AML | RARS |
| 1 | 0,434 | 0,002 | 0,002 | 0,000 | 0,018 | 0,044 | 0,000 | 0,628 | 0,007 | 0,001 | 0,956 |
| 2 | 0,089 | 0,074 | 0,000 | 0,000 | 0,495 | 0,065 | 0,001 | 0,783 | 0,016 | 0,003 | 0,913 |
| 3 | 0,006 | 0,189 | 0,000 | 0,000 | 0,991 | 0,293 | 0,000 | 0,676 | 0,008 | 0,002 | 0,827 |
| 4 | 0,001 | 0,054 | 0,000 | 0,000 | 0,957 | 0,362 | 0,001 | 0,397 | 0,009 | 0,003 | 0,827 |
| 5 | 0,001 | 0,000 | 0,000 | 0,000 | 0,631 | 0,289 | 0,002 | 0,652 | 0,015 | 0,009 | 0,935 |
| 6 | 0,003 | 0,000 | 0,000 | 0,000 | 0,162 | 0,174 | 0,002 | 0,939 | 0,029 | 0,021 | 0,414 |
| 7 | 0,009 | 0,001 | 0,000 | 0,000 | 0,100 | 0,091 | 0,011 | 0,869 | 0,169 | 0,106 | 0,430 |
| 8 | 0,023 | 0,000 | 0,001 | 0,001 | 0,056 | 0,131 | 0,019 | 0,741 | 0,329 | 0,155 | 0,479 |
| 9 | 0,062 | 0,002 | 0,001 | 0,002 | 0,060 | 0,073 | 0,056 | 0,956 | 0,541 | 0,466 | 0,461 |
| 10 | 0,036 | 0,001 | 0,000 | 0,004 | 0,052 | 0,022 | 0,099 | 0,708 | 0,833 | 0,873 | 0,605 |
| 11 | 0,053 | 0,000 | 0,000 | 0,001 | 0,004 | 0,004 | 0,017 | 0,921 | 0,774 | 0,735 | 0,957 |
| 12 | 0,024 | 0,001 | 0,000 | 0,000 | 0,042 | 0,000 | 0,002 | 0,071 | 0,169 | 0,845 | 0,605 |
| 13 | 0,046 | 0,129 | 0,000 | 0,000 | 0,306 | 0,000 | 0,002 | 0,137 | 0,104 | 0,649 | 0,487 |
| 14 | 0,073 | 0,294 | 0,000 | 0,000 | 0,447 | 0,000 | 0,005 | 0,091 | 0,063 | 0,726 | 1,000 |
| 15 | 0,174 | 0,156 | 0,000 | 0,000 | 0,290 | 0,000 | 0,003 | 0,071 | 0,055 | 0,804 | 0,685 |
| 16 | 0,747 | 0,003 | 0,000 | 0,003 | 0,008 | 0,001 | 0,006 | 0,940 | 0,252 | 0,470 | 0,862 |
| 17 | 0,628 | 0,001 | 0,001 | 0,003 | 0,002 | 0,003 | 0,008 | 0,930 | 0,633 | 0,577 | 1,000 |
| 18 | 0,805 | 0,001 | 0,007 | 0,010 | 0,001 | 0,007 | 0,009 | 0,554 | 0,834 | 0,495 | 0,839 |
| 19 | 0,766 | 0,001 | 0,003 | 0,014 | 0,004 | 0,021 | 0,022 | 0,579 | 0,954 | 0,469 | 1,000 |
| 20 | 0,405 | 0,003 | 0,015 | 0,020 | 0,040 | 0,128 | 0,075 | 0,481 | 0,674 | 0,397 | 0,931 |
| 21 | 0,270 | 0,009 | 0,099 | 0,016 | 0,090 | 0,604 | 0,091 | 0,371 | 0,554 | 0,223 | 0,885 |
| 22 | 0,153 | 0,031 | 0,251 | 0,013 | 0,239 | 0,795 | 0,098 | 0,371 | 0,302 | 0,112 | 0,908 |
| 23 | 0,124 | 0,003 | 0,033 | 0,006 | 0,075 | 0,631 | 0,092 | 0,422 | 0,411 | 0,150 | 0,663 |
| 24 | 0,089 | 0,004 | 0,014 | 0,020 | 0,080 | 0,308 | 0,204 | 0,741 | 0,541 | 0,683 | 0,935 |
| 25 | 0,156 | 0,006 | 0,009 | 0,036 | 0,105 | 0,155 | 0,229 | 0,930 | 0,541 | 0,670 | 0,913 |
| 26 | 0,045 | 0,006 | 0,000 | 0,019 | 0,316 | 0,016 | 0,286 | 0,538 | 0,646 | 1,000 | 0,827 |
| 27 | 0,035 | 0,023 | 0,000 | 0,001 | 0,291 | 0,003 | 0,012 | 0,311 | 0,131 | 0,434 | 0,663 |
| 28 | 0,009 | 0,000 | 0,000 | 0,001 | 0,042 | 0,004 | 0,011 | 0,560 | 0,175 | 0,214 | 0,785 |
| 29 | 0,002 | 0,000 | 0,000 | 0,000 | 0,011 | 0,001 | 0,007 | 0,560 | 0,113 | 0,201 | 0,355 |
| 30 | 0,011 | 0,000 | 0,000 | 0,000 | 0,002 | 0,001 | 0,004 | 0,817 | 0,109 | 0,114 | 0,586 |
| 31 | 0,001 | 0,000 | 0,000 | 0,002 | 0,003 | 0,001 | 0,022 | 0,991 | 0,133 | 0,207 | 0,479 |
| 32 | 0,027 | 0,000 | 0,000 | 0,001 | 0,007 | 0,000 | 0,005 | 0,545 | 0,064 | 0,188 | 0,586 |
| 33 | 0,024 | 0,010 | 0,000 | 0,001 | 0,094 | 0,000 | 0,006 | 0,322 | 0,048 | 0,166 | 0,242 |
| 34 | 0,022 | 0,007 | 0,000 | 0,000 | 0,078 | 0,000 | 0,010 | 0,468 | 0,130 | 0,263 | 0,097 |
| 35 | 0,086 | 0,014 | 0,000 | 0,000 | 0,074 | 0,001 | 0,009 | 0,441 | 0,213 | 0,320 | 0,148 |
| 36 | 0,091 | 0,000 | 0,000 | 0,000 | 0,004 | 0,001 | 0,007 | 0,461 | 0,434 | 0,631 | 0,072 |
| 37 | 0,014 | 0,000 | 0,000 | 0,001 | 0,000 | 0,002 | 0,023 | 0,750 | 0,804 | 0,972 | 0,036 |
| 38 | 0,041 | 0,000 | 0,000 | 0,003 | 0,001 | 0,001 | 0,044 | 0,523 | 0,954 | 0,887 | 0,101 |
| 39 | 0,406 | 0,000 | 0,000 | 0,001 | 0,000 | 0,000 | 0,006 | 0,448 | 0,834 | 1,000 | 0,190 |
| 40 | 0,476 | 0,002 | 0,001 | 0,001 | 0,012 | 0,006 | 0,004 | 0,481 | 0,169 | 0,434 | 0,237 |
| 41 | 0,565 | 0,243 | 0,014 | 0,011 | 0,077 | 0,008 | 0,004 | 0,355 | 0,146 | 0,374 | 0,564 |
| 42 | 0,344 | 0,506 | 0,024 | 0,016 | 0,179 | 0,004 | 0,004 | 0,204 | 0,075 | 0,455 | 0,583 |
| 43 | 0,124 | 0,023 | 0,000 | 0,026 | 0,179 | 0,001 | 0,130 | 0,226 | 0,479 | 0,790 | 0,566 |
| 44 | 0,153 | 0,001 | 0,000 | 0,011 | 0,022 | 0,001 | 0,082 | 0,409 | 0,660 | 0,986 | 0,567 |
| 45 | 0,242 | 0,000 | 0,000 | 0,016 | 0,003 | 0,000 | 0,071 | 0,636 | 0,660 | 0,915 | 0,190 |
| 46 | 0,200 | 0,000 | 0,000 | 0,001 | 0,004 | 0,000 | 0,008 | 1,000 | 0,718 | 0,845 | 0,050 |
| 47 | 0,681 | 0,000 | 0,000 | 0,000 | 0,001 | 0,000 | 0,001 | 0,919 | 0,278 | 0,258 | 0,018 |
| 48 | 0,416 | 0,000 | 0,000 | 0,000 | 0,000 | 0,000 | 0,000 | 0,919 | 0,232 | 0,151 | 0,141 |
| 49 | 0,607 | 0,000 | 0,000 | 0,000 | 0,000 | 0,000 | 0,000 | 0,869 | 0,201 | 0,145 | 0,134 |
| 50 | 0,797 | 0,000 | 0,000 | 0,000 | 0,001 | 0,001 | 0,000 | 0,970 | 0,090 | 0,170 | 0,200 |
| 51 | 0,640 | 0,002 | 0,000 | 0,000 | 0,011 | 0,002 | 0,001 | 0,638 | 0,141 | 0,128 | 0,683 |
| 52 | 0,981 | 0,073 | 0,002 | 0,000 | 0,223 | 0,003 | 0,002 | 0,367 | 0,141 | 0,201 | 0,314 |
| 53 | 1,000 | 0,248 | 0,004 | 0,000 | 0,359 | 0,010 | 0,000 | 0,344 | 0,025 | 0,031 | 0,806 |
| 54 | 0,766 | 0,538 | 0,068 | 0,000 | 0,557 | 0,040 | 0,000 | 0,531 | 0,023 | 0,013 | 0,604 |
| 55 | 0,629 | 0,826 | 0,703 | 0,003 | 0,572 | 1,000 | 0,004 | 0,652 | 0,039 | 0,006 | 0,806 |
| 56 | 0,541 | 0,641 | 0,667 | 0,001 | 0,462 | 0,984 | 0,002 | 0,560 | 0,039 | 0,002 | 1,000 |
| 57 | 0,376 | 0,958 | 0,285 | 0,002 | 0,399 | 0,764 | 0,002 | 0,468 | 0,027 | 0,001 | 0,703 |
| 58 | 0,231 | 0,937 | 0,126 | 0,002 | 0,332 | 0,904 | 0,001 | 0,355 | 0,029 | 0,001 | 0,496 |
| 59 | 0,148 | 0,937 | 0,264 | 0,001 | 0,197 | 0,849 | 0,000 | 0,301 | 0,014 | 0,001 | 0,568 |
| 60 | 0,072 | 0,909 | 0,177 | 0,001 | 0,200 | 0,756 | 0,000 | 0,301 | 0,009 | 0,001 | 0,414 |
| 61 | 0,159 | 0,840 | 0,371 | 0,000 | 0,208 | 0,582 | 0,000 | 0,350 | 0,003 | 0,000 | 0,683 |
| 62 | 0,207 | 0,819 | 0,389 | 0,000 | 0,176 | 0,423 | 0,000 | 0,403 | 0,002 | 0,000 | 0,892 |
| 63 | 0,201 | 0,758 | 0,508 | 0,000 | 0,183 | 0,357 | 0,000 | 0,575 | 0,001 | 0,000 | 0,913 |
| 64 | 0,323 | 0,503 | 1,000 | 0,000 | 0,141 | 0,342 | 0,000 | 0,350 | 0,000 | 0,000 | 0,892 |
| 65 | 0,307 | 0,275 | 0,466 | 0,000 | 0,073 | 0,180 | 0,000 | 0,516 | 0,001 | 0,000 | 0,935 |
| Statistically significant p-values (≤ 0.05) are highlighted in yellow | | | | | | | | | | | |

| **Table S4. Statistical results** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| window start point | control | control | control | control | low risk | low risk | low risk | RAEB | RAEB | CMML | RA |
| (window size = 5) | vs. | vs. | vs. | vs. | vs. | vs. | vs. | vs. | vs. | vs. | vs. |
| | low risk | RAEB | CMML | AML | RAEB | CMML | AML | CMML | AML | AML | RARS |
| 1 | 0,096 | 0,008 | 0,000 | 0,000 | 0,119 | 0,034 | 0,000 | 1,000 | 0,013 | 0,002 | 0,827 |
| 2 | 0,012 | 0,098 | 0,000 | 0,000 | 0,831 | 0,121 | 0,000 | 0,708 | 0,013 | 0,003 | 0,828 |
| 3 | 0,003 | 0,084 | 0,000 | 0,000 | 0,923 | 0,280 | 0,001 | 0,660 | 0,010 | 0,002 | 0,744 |
| 4 | 0,000 | 0,002 | 0,000 | 0,000 | 0,798 | 0,317 | 0,002 | 0,560 | 0,011 | 0,007 | 0,913 |
| 5 | 0,001 | 0,000 | 0,000 | 0,000 | 0,326 | 0,171 | 0,002 | 0,930 | 0,032 | 0,014 | 0,724 |
| 6 | 0,004 | 0,001 | 0,000 | 0,000 | 0,208 | 0,153 | 0,004 | 0,886 | 0,054 | 0,029 | 0,446 |
| 7 | 0,010 | 0,001 | 0,000 | 0,000 | 0,094 | 0,147 | 0,010 | 0,758 | 0,175 | 0,091 | 0,462 |
| 8 | 0,016 | 0,000 | 0,000 | 0,001 | 0,044 | 0,093 | 0,032 | 0,852 | 0,411 | 0,294 | 0,327 |
| 9 | 0,068 | 0,001 | 0,000 | 0,004 | 0,024 | 0,016 | 0,054 | 0,965 | 0,848 | 0,696 | 0,462 |
| 10 | 0,035 | 0,001 | 0,000 | 0,002 | 0,037 | 0,010 | 0,052 | 0,613 | 0,606 | 0,696 | 0,870 |
| 11 | 0,033 | 0,000 | 0,000 | 0,000 | 0,027 | 0,000 | 0,002 | 0,206 | 0,194 | 0,749 | 0,849 |
| 12 | 0,008 | 0,022 | 0,000 | 0,000 | 0,258 | 0,000 | 0,004 | 0,082 | 0,126 | 0,722 | 0,785 |
| 13 | 0,110 | 0,206 | 0,000 | 0,001 | 0,333 | 0,000 | 0,004 | 0,078 | 0,093 | 0,901 | 0,772 |
| 14 | 0,177 | 0,134 | 0,000 | 0,000 | 0,290 | 0,000 | 0,002 | 0,106 | 0,063 | 0,695 | 0,622 |
| 15 | 0,425 | 0,004 | 0,000 | 0,000 | 0,010 | 0,000 | 0,001 | 0,278 | 0,121 | 0,563 | 0,643 |
| 16 | 0,410 | 0,002 | 0,000 | 0,003 | 0,006 | 0,002 | 0,012 | 0,990 | 0,330 | 0,509 | 0,931 |
| 17 | 0,932 | 0,002 | 0,002 | 0,007 | 0,002 | 0,003 | 0,007 | 0,870 | 0,688 | 0,665 | 0,862 |
| 18 | 0,926 | 0,001 | 0,004 | 0,011 | 0,002 | 0,013 | 0,011 | 0,562 | 0,849 | 0,495 | 0,794 |
| 19 | 0,524 | 0,001 | 0,009 | 0,008 | 0,011 | 0,079 | 0,026 | 0,457 | 0,775 | 0,386 | 1,000 |
| 20 | 0,285 | 0,004 | 0,039 | 0,018 | 0,062 | 0,423 | 0,085 | 0,427 | 0,688 | 0,322 | 0,931 |
| 21 | 0,304 | 0,018 | 0,180 | 0,015 | 0,113 | 0,724 | 0,070 | 0,302 | 0,379 | 0,186 | 0,750 |
| 22 | 0,200 | 0,006 | 0,016 | 0,009 | 0,077 | 0,384 | 0,086 | 0,621 | 0,401 | 0,286 | 0,643 |
| 23 | 0,075 | 0,002 | 0,012 | 0,015 | 0,068 | 0,271 | 0,169 | 0,766 | 0,516 | 0,582 | 0,870 |
| 24 | 0,077 | 0,003 | 0,013 | 0,019 | 0,096 | 0,308 | 0,236 | 0,700 | 0,592 | 0,696 | 0,936 |
| 25 | 0,039 | 0,003 | 0,001 | 0,013 | 0,186 | 0,032 | 0,154 | 0,792 | 0,468 | 0,736 | 0,683 |
| 26 | 0,057 | 0,011 | 0,000 | 0,001 | 0,153 | 0,003 | 0,015 | 0,435 | 0,260 | 0,455 | 0,935 |
| 27 | 0,010 | 0,000 | 0,000 | 0,001 | 0,092 | 0,005 | 0,021 | 0,488 | 0,201 | 0,346 | 0,913 |
| 28 | 0,003 | 0,000 | 0,000 | 0,001 | 0,018 | 0,002 | 0,007 | 0,676 | 0,152 | 0,220 | 0,354 |
| 29 | 0,003 | 0,000 | 0,000 | 0,000 | 0,011 | 0,001 | 0,008 | 0,582 | 0,109 | 0,177 | 0,429 |
| 30 | 0,005 | 0,000 | 0,000 | 0,000 | 0,006 | 0,000 | 0,003 | 0,676 | 0,082 | 0,122 | 0,514 |
| 31 | 0,001 | 0,000 | 0,000 | 0,001 | 0,002 | 0,001 | 0,015 | 0,991 | 0,122 | 0,183 | 0,355 |
| 32 | 0,030 | 0,002 | 0,000 | 0,001 | 0,037 | 0,000 | 0,005 | 0,322 | 0,056 | 0,155 | 0,242 |
| 33 | 0,017 | 0,011 | 0,000 | 0,000 | 0,073 | 0,000 | 0,004 | 0,422 | 0,078 | 0,213 | 0,191 |
| 34 | 0,036 | 0,010 | 0,000 | 0,000 | 0,082 | 0,001 | 0,013 | 0,481 | 0,151 | 0,320 | 0,165 |
| 35 | 0,059 | 0,000 | 0,000 | 0,000 | 0,002 | 0,000 | 0,004 | 0,567 | 0,369 | 0,499 | 0,047 |
| 36 | 0,029 | 0,000 | 0,000 | 0,000 | 0,001 | 0,001 | 0,010 | 0,428 | 0,422 | 0,817 | 0,077 |
| 37 | 0,018 | 0,000 | 0,000 | 0,001 | 0,000 | 0,002 | 0,023 | 0,733 | 0,863 | 1,000 | 0,060 |
| 38 | 0,346 | 0,000 | 0,000 | 0,001 | 0,000 | 0,001 | 0,008 | 0,385 | 0,804 | 0,972 | 0,156 |
| 39 | 0,206 | 0,000 | 0,000 | 0,001 | 0,001 | 0,001 | 0,007 | 0,428 | 0,674 | 0,901 | 0,091 |
| 40 | 0,783 | 0,005 | 0,002 | 0,002 | 0,014 | 0,007 | 0,005 | 0,435 | 0,194 | 0,445 | 0,249 |
| 41 | 0,340 | 0,301 | 0,019 | 0,017 | 0,075 | 0,005 | 0,004 | 0,243 | 0,092 | 0,477 | 0,640 |
| 42 | 0,664 | 0,048 | 0,000 | 0,011 | 0,105 | 0,000 | 0,023 | 0,162 | 0,293 | 1,000 | 0,703 |
| 43 | 0,054 | 0,004 | 0,000 | 0,009 | 0,084 | 0,002 | 0,106 | 0,367 | 0,553 | 0,929 | 0,978 |
| 44 | 0,284 | 0,000 | 0,000 | 0,012 | 0,007 | 0,000 | 0,061 | 0,441 | 0,592 | 0,972 | 0,252 |
| 45 | 0,215 | 0,000 | 0,000 | 0,001 | 0,002 | 0,000 | 0,006 | 0,741 | 0,390 | 0,582 | 0,064 |
| 46 | 0,717 | 0,000 | 0,000 | 0,000 | 0,001 | 0,000 | 0,001 | 0,909 | 0,470 | 0,617 | 0,017 |
| 47 | 0,729 | 0,000 | 0,000 | 0,000 | 0,000 | 0,000 | 0,000 | 0,889 | 0,278 | 0,170 | 0,041 |
| 48 | 0,479 | 0,000 | 0,000 | 0,000 | 0,000 | 0,000 | 0,000 | 0,929 | 0,201 | 0,133 | 0,102 |
| 49 | 0,974 | 0,000 | 0,000 | 0,000 | 0,000 | 0,000 | 0,000 | 0,959 | 0,141 | 0,164 | 0,191 |
| 50 | 0,910 | 0,001 | 0,000 | 0,000 | 0,004 | 0,001 | 0,000 | 0,770 | 0,141 | 0,157 | 0,354 |
| 51 | 0,910 | 0,024 | 0,001 | 0,000 | 0,124 | 0,002 | 0,001 | 0,373 | 0,169 | 0,255 | 0,764 |
| 52 | 0,942 | 0,075 | 0,005 | 0,000 | 0,193 | 0,010 | 0,000 | 0,409 | 0,043 | 0,057 | 0,723 |
| 53 | 0,772 | 0,298 | 0,007 | 0,000 | 0,405 | 0,016 | 0,000 | 0,416 | 0,015 | 0,023 | 0,892 |
| 54 | 0,847 | 0,660 | 0,226 | 0,000 | 0,536 | 0,147 | 0,000 | 0,758 | 0,027 | 0,007 | 0,828 |
| 55 | 0,541 | 0,673 | 0,722 | 0,001 | 0,550 | 0,984 | 0,002 | 0,560 | 0,032 | 0,003 | 0,849 |
| 56 | 0,398 | 0,771 | 0,456 | 0,001 | 0,394 | 0,920 | 0,002 | 0,422 | 0,031 | 0,002 | 0,849 |
| 57 | 0,215 | 0,965 | 0,185 | 0,002 | 0,321 | 0,889 | 0,001 | 0,403 | 0,034 | 0,002 | 0,605 |
| 58 | 0,172 | 1,000 | 0,182 | 0,001 | 0,281 | 0,992 | 0,000 | 0,356 | 0,017 | 0,001 | 0,496 |
| 59 | 0,113 | 0,888 | 0,305 | 0,001 | 0,186 | 0,779 | 0,000 | 0,291 | 0,014 | 0,001 | 0,496 |
| 60 | 0,150 | 0,944 | 0,289 | 0,001 | 0,228 | 0,696 | 0,000 | 0,385 | 0,003 | 0,000 | 0,663 |
| 61 | 0,128 | 0,937 | 0,289 | 0,001 | 0,159 | 0,453 | 0,000 | 0,350 | 0,004 | 0,000 | 0,744 |
| 62 | 0,195 | 0,725 | 0,518 | 0,000 | 0,162 | 0,327 | 0,000 | 0,509 | 0,001 | 0,000 | 1,000 |
| 63 | 0,296 | 0,526 | 0,862 | 0,000 | 0,144 | 0,317 | 0,000 | 0,403 | 0,000 | 0,000 | 0,935 |
| 64 | 0,376 | 0,316 | 0,697 | 0,000 | 0,101 | 0,284 | 0,000 | 0,344 | 0,001 | 0,000 | 0,935 |
| Statistically significant p-values (≤ 0.05) are highlighted in yellow | | | | | | | | | | | |

| **Table S5. Statistical results** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| window start point | control | control | control | control | low risk | low risk | low risk | RAEB | RAEB | CMML | RA |
| (window size = 6) | vs. | vs. | vs. | vs. | vs. | vs. | vs. | vs. | vs. | vs. | vs. |
| | low risk | RAEB | CMML | AML | RAEB | CMML | AML | CMML | AML | AML | RARS |
| 1 | 0,019 | 0,020 | 0,000 | 0,000 | 0,399 | 0,080 | 0,000 | 0,758 | 0,010 | 0,002 | 0,913 |
| 2 | 0,006 | 0,048 | 0,000 | 0,000 | 0,757 | 0,167 | 0,001 | 0,700 | 0,010 | 0,002 | 0,744 |
| 3 | 0,002 | 0,003 | 0,000 | 0,000 | 0,693 | 0,267 | 0,001 | 0,700 | 0,010 | 0,004 | 0,807 |
| 4 | 0,001 | 0,001 | 0,000 | 0,000 | 0,400 | 0,215 | 0,002 | 0,783 | 0,021 | 0,009 | 0,644 |
| 5 | 0,002 | 0,001 | 0,000 | 0,000 | 0,359 | 0,193 | 0,002 | 0,758 | 0,045 | 0,019 | 0,605 |
| 6 | 0,006 | 0,001 | 0,000 | 0,000 | 0,165 | 0,187 | 0,004 | 0,974 | 0,067 | 0,033 | 0,479 |
| 7 | 0,007 | 0,000 | 0,000 | 0,001 | 0,080 | 0,109 | 0,013 | 0,965 | 0,251 | 0,183 | 0,369 |
| 8 | 0,017 | 0,000 | 0,000 | 0,001 | 0,020 | 0,025 | 0,029 | 0,886 | 0,606 | 0,414 | 0,430 |
| 9 | 0,079 | 0,001 | 0,000 | 0,003 | 0,019 | 0,006 | 0,032 | 0,800 | 0,567 | 0,696 | 0,764 |
| 10 | 0,030 | 0,001 | 0,000 | 0,000 | 0,073 | 0,001 | 0,008 | 0,156 | 0,236 | 0,859 | 0,978 |
| 11 | 0,014 | 0,004 | 0,000 | 0,000 | 0,144 | 0,000 | 0,005 | 0,206 | 0,169 | 0,606 | 1,000 |
| 12 | 0,030 | 0,109 | 0,000 | 0,000 | 0,326 | 0,000 | 0,005 | 0,053 | 0,117 | 0,915 | 1,000 |
| 13 | 0,197 | 0,091 | 0,000 | 0,000 | 0,159 | 0,000 | 0,003 | 0,096 | 0,105 | 0,773 | 0,839 |
| 14 | 0,359 | 0,003 | 0,000 | 0,000 | 0,009 | 0,000 | 0,001 | 0,203 | 0,117 | 0,409 | 0,728 |
| 15 | 0,259 | 0,002 | 0,000 | 0,000 | 0,014 | 0,000 | 0,004 | 0,320 | 0,207 | 0,664 | 0,794 |
| 16 | 0,766 | 0,003 | 0,000 | 0,005 | 0,006 | 0,002 | 0,011 | 0,980 | 0,349 | 0,563 | 0,772 |
| 17 | 0,926 | 0,002 | 0,002 | 0,006 | 0,004 | 0,004 | 0,010 | 0,900 | 0,674 | 0,665 | 0,954 |
| 18 | 0,684 | 0,001 | 0,007 | 0,008 | 0,006 | 0,058 | 0,016 | 0,521 | 0,731 | 0,409 | 0,954 |
| 19 | 0,368 | 0,002 | 0,023 | 0,008 | 0,023 | 0,179 | 0,029 | 0,413 | 0,688 | 0,302 | 1,000 |
| 20 | 0,308 | 0,008 | 0,109 | 0,016 | 0,065 | 0,548 | 0,073 | 0,314 | 0,480 | 0,223 | 0,954 |
| 21 | 0,338 | 0,004 | 0,016 | 0,012 | 0,039 | 0,123 | 0,059 | 0,560 | 0,422 | 0,355 | 0,604 |
| 22 | 0,084 | 0,002 | 0,006 | 0,015 | 0,079 | 0,180 | 0,126 | 0,843 | 0,554 | 0,631 | 0,807 |
| 23 | 0,091 | 0,001 | 0,012 | 0,015 | 0,070 | 0,276 | 0,198 | 0,613 | 0,529 | 0,594 | 0,724 |
| 24 | 0,023 | 0,001 | 0,001 | 0,008 | 0,114 | 0,128 | 0,204 | 0,912 | 0,504 | 0,709 | 0,703 |
| 25 | 0,063 | 0,003 | 0,000 | 0,001 | 0,147 | 0,009 | 0,009 | 0,553 | 0,181 | 0,320 | 0,892 |
| 26 | 0,018 | 0,000 | 0,000 | 0,001 | 0,041 | 0,003 | 0,022 | 0,516 | 0,268 | 0,384 | 0,935 |
| 27 | 0,003 | 0,000 | 0,000 | 0,001 | 0,020 | 0,002 | 0,012 | 0,598 | 0,154 | 0,294 | 0,549 |
| 28 | 0,005 | 0,000 | 0,000 | 0,001 | 0,016 | 0,001 | 0,008 | 0,652 | 0,122 | 0,201 | 0,513 |
| 29 | 0,002 | 0,000 | 0,000 | 0,000 | 0,020 | 0,001 | 0,006 | 0,482 | 0,105 | 0,183 | 0,414 |
| 30 | 0,005 | 0,000 | 0,000 | 0,000 | 0,004 | 0,000 | 0,003 | 0,652 | 0,069 | 0,106 | 0,327 |
| 31 | 0,002 | 0,000 | 0,000 | 0,001 | 0,007 | 0,000 | 0,011 | 0,509 | 0,073 | 0,195 | 0,221 |
| 32 | 0,027 | 0,002 | 0,000 | 0,001 | 0,031 | 0,000 | 0,004 | 0,441 | 0,086 | 0,171 | 0,142 |
| 33 | 0,027 | 0,015 | 0,000 | 0,000 | 0,086 | 0,001 | 0,007 | 0,468 | 0,099 | 0,278 | 0,242 |
| 34 | 0,017 | 0,000 | 0,000 | 0,000 | 0,003 | 0,000 | 0,005 | 0,428 | 0,158 | 0,337 | 0,053 |
| 35 | 0,020 | 0,000 | 0,000 | 0,000 | 0,000 | 0,000 | 0,006 | 0,524 | 0,401 | 0,722 | 0,039 |
| 36 | 0,030 | 0,000 | 0,000 | 0,000 | 0,001 | 0,001 | 0,012 | 0,441 | 0,422 | 0,776 | 0,086 |
| 37 | 0,159 | 0,000 | 0,000 | 0,000 | 0,000 | 0,001 | 0,004 | 0,567 | 0,879 | 0,803 | 0,060 |
| 38 | 0,215 | 0,000 | 0,000 | 0,001 | 0,001 | 0,002 | 0,010 | 0,435 | 0,819 | 0,957 | 0,091 |
| 39 | 0,288 | 0,000 | 0,000 | 0,002 | 0,001 | 0,001 | 0,009 | 0,403 | 0,660 | 0,845 | 0,157 |
| 40 | 0,872 | 0,011 | 0,002 | 0,004 | 0,013 | 0,005 | 0,005 | 0,488 | 0,146 | 0,477 | 0,365 |
| 41 | 0,693 | 0,037 | 0,000 | 0,015 | 0,065 | 0,000 | 0,028 | 0,187 | 0,320 | 1,000 | 0,702 |
| 42 | 0,426 | 0,011 | 0,000 | 0,004 | 0,052 | 0,001 | 0,030 | 0,311 | 0,390 | 0,929 | 1,000 |
| 43 | 0,161 | 0,001 | 0,000 | 0,007 | 0,029 | 0,001 | 0,074 | 0,306 | 0,606 | 0,887 | 0,682 |
| 44 | 0,266 | 0,000 | 0,000 | 0,001 | 0,001 | 0,000 | 0,008 | 0,575 | 0,433 | 0,657 | 0,127 |
| 45 | 0,910 | 0,000 | 0,000 | 0,000 | 0,000 | 0,000 | 0,002 | 0,553 | 0,302 | 0,445 | 0,019 |
| 46 | 0,828 | 0,000 | 0,000 | 0,000 | 0,001 | 0,000 | 0,000 | 0,919 | 0,420 | 0,472 | 0,044 |
| 47 | 0,682 | 0,000 | 0,000 | 0,000 | 0,000 | 0,000 | 0,000 | 0,909 | 0,254 | 0,184 | 0,027 |
| 48 | 0,772 | 0,000 | 0,000 | 0,000 | 0,000 | 0,000 | 0,000 | 0,949 | 0,141 | 0,157 | 0,134 |
| 49 | 0,949 | 0,000 | 0,000 | 0,000 | 0,002 | 0,001 | 0,000 | 0,980 | 0,156 | 0,164 | 0,341 |
| 50 | 0,735 | 0,015 | 0,001 | 0,000 | 0,040 | 0,001 | 0,001 | 0,461 | 0,169 | 0,346 | 0,369 |
| 51 | 0,968 | 0,025 | 0,002 | 0,000 | 0,082 | 0,004 | 0,000 | 0,468 | 0,064 | 0,060 | 1,000 |
| 52 | 0,828 | 0,093 | 0,008 | 0,000 | 0,169 | 0,013 | 0,000 | 0,488 | 0,034 | 0,024 | 0,935 |
| 53 | 0,784 | 0,408 | 0,025 | 0,000 | 0,449 | 0,075 | 0,000 | 0,560 | 0,031 | 0,016 | 0,892 |
| 54 | 0,822 | 0,413 | 0,349 | 0,000 | 0,482 | 0,180 | 0,000 | 0,965 | 0,019 | 0,004 | 0,849 |
| 55 | 0,398 | 0,778 | 0,518 | 0,002 | 0,442 | 0,904 | 0,001 | 0,488 | 0,036 | 0,002 | 0,744 |
| 56 | 0,257 | 0,916 | 0,316 | 0,002 | 0,296 | 0,904 | 0,001 | 0,416 | 0,031 | 0,002 | 0,744 |
| 57 | 0,182 | 1,000 | 0,230 | 0,001 | 0,263 | 0,968 | 0,001 | 0,391 | 0,024 | 0,001 | 0,496 |
| 58 | 0,145 | 0,923 | 0,240 | 0,001 | 0,236 | 0,968 | 0,000 | 0,312 | 0,019 | 0,001 | 0,463 |
| 59 | 0,207 | 0,653 | 0,380 | 0,000 | 0,197 | 0,704 | 0,000 | 0,306 | 0,006 | 0,000 | 0,807 |
| 60 | 0,119 | 0,965 | 0,263 | 0,001 | 0,197 | 0,555 | 0,000 | 0,350 | 0,004 | 0,000 | 0,786 |
| 61 | 0,128 | 0,860 | 0,398 | 0,000 | 0,169 | 0,418 | 0,000 | 0,475 | 0,002 | 0,000 | 0,892 |
| 62 | 0,278 | 0,515 | 0,855 | 0,000 | 0,127 | 0,271 | 0,000 | 0,461 | 0,000 | 0,000 | 0,935 |
| 63 | 0,347 | 0,374 | 0,855 | 0,000 | 0,105 | 0,298 | 0,000 | 0,350 | 0,001 | 0,000 | 1,000 |
| Statistically significant p-values (< 0.05) are highlighted in yellow | | | | | | | | | | | |

**Table S6. Statistical results**

| window start point (window size = 7) | control vs. low risk | control vs. RAEB | control vs. CMML | control vs. AML | low risk vs. RAEB | low risk vs. CMML | low risk vs. AML | RAEB vs. CMML | RAEB vs. AML | CMML vs. AML | RA vs. RARS |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0,009 | 0,016 | 0,000 | 0,000 | 0,482 | 0,077 | 0,000 | 0,947 | 0,009 | 0,001 | 0,870 |
| 2 | 0,002 | 0,002 | 0,000 | 0,000 | 0,424 | 0,208 | 0,001 | 0,843 | 0,011 | 0,004 | 0,703 |
| 3 | 0,003 | 0,002 | 0,000 | 0,000 | 0,342 | 0,193 | 0,001 | 0,826 | 0,014 | 0,006 | 0,586 |
| 4 | 0,001 | 0,003 | 0,000 | 0,000 | 0,495 | 0,215 | 0,002 | 0,676 | 0,029 | 0,013 | 0,568 |
| 5 | 0,002 | 0,001 | 0,000 | 0,000 | 0,326 | 0,204 | 0,002 | 0,860 | 0,049 | 0,022 | 0,531 |
| 6 | 0,006 | 0,001 | 0,000 | 0,000 | 0,124 | 0,150 | 0,005 | 0,930 | 0,082 | 0,051 | 0,384 |
| 7 | 0,008 | 0,000 | 0,000 | 0,001 | 0,030 | 0,040 | 0,014 | 0,878 | 0,302 | 0,271 | 0,430 |
| 8 | 0,026 | 0,000 | 0,000 | 0,001 | 0,014 | 0,012 | 0,017 | 0,904 | 0,492 | 0,414 | 0,605 |
| 9 | 0,050 | 0,001 | 0,000 | 0,000 | 0,028 | 0,000 | 0,007 | 0,271 | 0,268 | 0,736 | 0,957 |
| 10 | 0,022 | 0,005 | 0,000 | 0,000 | 0,236 | 0,000 | 0,010 | 0,198 | 0,152 | 0,670 | 0,913 |
| 11 | 0,031 | 0,037 | 0,000 | 0,000 | 0,232 | 0,000 | 0,006 | 0,118 | 0,136 | 0,831 | 0,786 |
| 12 | 0,057 | 0,045 | 0,000 | 0,000 | 0,144 | 0,000 | 0,004 | 0,063 | 0,113 | 0,845 | 0,764 |
| 13 | 0,435 | 0,001 | 0,000 | 0,000 | 0,004 | 0,000 | 0,002 | 0,217 | 0,181 | 0,577 | 0,954 |
| 14 | 0,235 | 0,003 | 0,000 | 0,000 | 0,011 | 0,000 | 0,003 | 0,262 | 0,188 | 0,549 | 0,839 |
| 15 | 0,536 | 0,003 | 0,000 | 0,001 | 0,006 | 0,000 | 0,003 | 0,562 | 0,311 | 0,679 | 0,685 |
| 16 | 0,641 | 0,004 | 0,000 | 0,005 | 0,011 | 0,003 | 0,017 | 0,940 | 0,380 | 0,536 | 0,794 |
| 17 | 0,628 | 0,002 | 0,003 | 0,006 | 0,009 | 0,017 | 0,013 | 0,811 | 0,529 | 0,536 | 0,908 |
| 18 | 0,420 | 0,002 | 0,014 | 0,007 | 0,013 | 0,137 | 0,028 | 0,496 | 0,674 | 0,353 | 1,000 |
| 19 | 0,410 | 0,003 | 0,068 | 0,010 | 0,025 | 0,294 | 0,032 | 0,296 | 0,611 | 0,274 | 1,000 |
| 20 | 0,326 | 0,002 | 0,010 | 0,012 | 0,022 | 0,107 | 0,057 | 0,488 | 0,516 | 0,374 | 0,785 |
| 21 | 0,150 | 0,001 | 0,006 | 0,018 | 0,039 | 0,101 | 0,118 | 0,878 | 0,633 | 0,644 | 0,828 |
| 22 | 0,113 | 0,002 | 0,007 | 0,017 | 0,062 | 0,174 | 0,164 | 0,692 | 0,661 | 0,657 | 0,683 |
| 23 | 0,037 | 0,001 | 0,002 | 0,006 | 0,075 | 0,153 | 0,159 | 0,800 | 0,462 | 0,606 | 0,538 |
| 24 | 0,039 | 0,002 | 0,000 | 0,001 | 0,067 | 0,023 | 0,017 | 0,733 | 0,260 | 0,270 | 0,683 |
| 25 | 0,022 | 0,000 | 0,000 | 0,001 | 0,033 | 0,008 | 0,012 | 0,644 | 0,175 | 0,286 | 0,807 |
| 26 | 0,008 | 0,000 | 0,000 | 0,001 | 0,014 | 0,002 | 0,013 | 0,605 | 0,207 | 0,320 | 0,744 |
| 27 | 0,005 | 0,000 | 0,000 | 0,001 | 0,024 | 0,002 | 0,011 | 0,560 | 0,175 | 0,294 | 0,663 |
| 28 | 0,003 | 0,000 | 0,000 | 0,001 | 0,025 | 0,001 | 0,006 | 0,644 | 0,117 | 0,207 | 0,446 |
| 29 | 0,002 | 0,000 | 0,000 | 0,000 | 0,011 | 0,000 | 0,006 | 0,531 | 0,089 | 0,177 | 0,231 |
| 30 | 0,005 | 0,000 | 0,000 | 0,000 | 0,008 | 0,000 | 0,003 | 0,509 | 0,076 | 0,171 | 0,201 |
| 31 | 0,001 | 0,000 | 0,000 | 0,001 | 0,006 | 0,000 | 0,008 | 0,684 | 0,093 | 0,214 | 0,128 |
| 32 | 0,035 | 0,002 | 0,000 | 0,001 | 0,039 | 0,000 | 0,007 | 0,495 | 0,089 | 0,200 | 0,191 |
| 33 | 0,016 | 0,000 | 0,000 | 0,000 | 0,006 | 0,000 | 0,003 | 0,403 | 0,117 | 0,303 | 0,086 |
| 34 | 0,006 | 0,000 | 0,000 | 0,000 | 0,001 | 0,000 | 0,004 | 0,367 | 0,194 | 0,511 | 0,039 |
| 35 | 0,026 | 0,000 | 0,000 | 0,000 | 0,001 | 0,001 | 0,007 | 0,676 | 0,380 | 0,696 | 0,050 |
| 36 | 0,137 | 0,000 | 0,000 | 0,000 | 0,001 | 0,001 | 0,006 | 0,422 | 0,293 | 0,644 | 0,115 |
| 37 | 0,082 | 0,000 | 0,000 | 0,001 | 0,001 | 0,003 | 0,006 | 0,598 | 0,864 | 0,859 | 0,057 |
| 38 | 0,260 | 0,000 | 0,000 | 0,001 | 0,001 | 0,001 | 0,012 | 0,435 | 0,688 | 0,845 | 0,127 |
| 39 | 0,579 | 0,000 | 0,000 | 0,002 | 0,000 | 0,001 | 0,007 | 0,416 | 0,567 | 0,776 | 0,220 |
| 40 | 0,376 | 0,001 | 0,000 | 0,005 | 0,014 | 0,000 | 0,009 | 0,257 | 0,480 | 1,000 | 0,513 |
| 41 | 0,445 | 0,006 | 0,000 | 0,006 | 0,047 | 0,000 | 0,029 | 0,317 | 0,359 | 0,957 | 1,000 |
| 42 | 0,629 | 0,003 | 0,000 | 0,004 | 0,019 | 0,000 | 0,021 | 0,231 | 0,411 | 0,657 | 0,663 |
| 43 | 0,113 | 0,000 | 0,000 | 0,001 | 0,004 | 0,000 | 0,006 | 0,538 | 0,401 | 0,619 | 0,369 |
| 44 | 0,872 | 0,000 | 0,000 | 0,000 | 0,001 | 0,000 | 0,002 | 0,567 | 0,349 | 0,522 | 0,050 |
| 45 | 0,994 | 0,000 | 0,000 | 0,000 | 0,000 | 0,000 | 0,001 | 0,598 | 0,222 | 0,374 | 0,047 |
| 46 | 0,981 | 0,000 | 0,000 | 0,000 | 0,001 | 0,000 | 0,000 | 0,899 | 0,304 | 0,338 | 0,029 |
| 47 | 0,987 | 0,000 | 0,000 | 0,000 | 0,001 | 0,000 | 0,000 | 0,950 | 0,243 | 0,177 | 0,108 |
| 48 | 0,910 | 0,000 | 0,000 | 0,000 | 0,001 | 0,000 | 0,000 | 0,839 | 0,148 | 0,157 | 0,211 |
| 49 | 0,917 | 0,006 | 0,000 | 0,000 | 0,027 | 0,001 | 0,001 | 0,582 | 0,201 | 0,394 | 0,355 |
| 50 | 0,711 | 0,015 | 0,001 | 0,000 | 0,041 | 0,002 | 0,000 | 0,448 | 0,059 | 0,065 | 0,723 |
| 51 | 0,822 | 0,042 | 0,002 | 0,000 | 0,080 | 0,005 | 0,000 | 0,516 | 0,049 | 0,048 | 0,892 |
| 52 | 0,828 | 0,208 | 0,017 | 0,000 | 0,267 | 0,034 | 0,000 | 0,482 | 0,034 | 0,019 | 0,663 |
| 53 | 0,942 | 0,369 | 0,072 | 0,000 | 0,424 | 0,091 | 0,000 | 0,668 | 0,029 | 0,008 | 1,000 |
| 54 | 0,618 | 0,498 | 0,613 | 0,000 | 0,412 | 0,332 | 0,000 | 0,886 | 0,017 | 0,003 | 0,806 |
| 55 | 0,267 | 0,888 | 0,376 | 0,002 | 0,348 | 0,897 | 0,001 | 0,468 | 0,041 | 0,001 | 0,644 |
| 56 | 0,219 | 0,895 | 0,403 | 0,001 | 0,241 | 1,000 | 0,001 | 0,468 | 0,027 | 0,001 | 0,643 |
| 57 | 0,152 | 0,951 | 0,278 | 0,001 | 0,224 | 0,984 | 0,000 | 0,385 | 0,021 | 0,001 | 0,462 |
| 58 | 0,182 | 0,685 | 0,362 | 0,001 | 0,245 | 0,865 | 0,000 | 0,322 | 0,009 | 0,000 | 0,568 |
| 59 | 0,133 | 0,692 | 0,362 | 0,000 | 0,169 | 0,660 | 0,000 | 0,328 | 0,005 | 0,000 | 0,807 |
| 60 | 0,126 | 0,881 | 0,354 | 0,000 | 0,169 | 0,503 | 0,000 | 0,435 | 0,004 | 0,000 | 0,807 |
| 61 | 0,237 | 0,520 | 0,716 | 0,000 | 0,130 | 0,285 | 0,000 | 0,385 | 0,002 | 0,000 | 1,000 |
| 62 | 0,351 | 0,393 | 0,960 | 0,000 | 0,101 | 0,222 | 0,000 | 0,422 | 0,001 | 0,000 | 0,978 |

Statistically significant p-values (< 0.05) are highlighted in yellow

**Table S7. Statistical results**

| window start point (window size = 8) | control vs. low risk | control vs. RAEB | control vs. CMML | control vs. AML | low risk vs. RAEB | low risk vs. CMML | low risk vs. AML | RAEB vs. CMML | RAEB vs. AML | CMML vs. AML | RA vs. RARS |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0,003 | 0,001 | 0,000 | 0,000 | 0,267 | 0,109 | 0,000 | 0,878 | 0,009 | 0,002 | 0,828 |
| 2 | 0,004 | 0,002 | 0,000 | 0,000 | 0,258 | 0,155 | 0,001 | 0,965 | 0,013 | 0,005 | 0,549 |
| 3 | 0,003 | 0,004 | 0,000 | 0,000 | 0,388 | 0,153 | 0,001 | 0,750 | 0,021 | 0,010 | 0,514 |
| 4 | 0,002 | 0,002 | 0,000 | 0,000 | 0,436 | 0,208 | 0,002 | 0,809 | 0,039 | 0,020 | 0,531 |
| 5 | 0,003 | 0,001 | 0,000 | 0,000 | 0,263 | 0,164 | 0,003 | 0,912 | 0,043 | 0,041 | 0,514 |
| 6 | 0,005 | 0,000 | 0,000 | 0,000 | 0,092 | 0,050 | 0,005 | 0,895 | 0,089 | 0,110 | 0,355 |
| 7 | 0,011 | 0,000 | 0,000 | 0,000 | 0,017 | 0,017 | 0,009 | 0,947 | 0,302 | 0,271 | 0,514 |
| 8 | 0,016 | 0,000 | 0,000 | 0,000 | 0,014 | 0,001 | 0,006 | 0,448 | 0,244 | 0,511 | 0,663 |
| 9 | 0,060 | 0,003 | 0,000 | 0,000 | 0,107 | 0,000 | 0,009 | 0,248 | 0,188 | 0,619 | 0,724 |
| 10 | 0,052 | 0,023 | 0,000 | 0,000 | 0,311 | 0,000 | 0,011 | 0,097 | 0,126 | 0,776 | 0,870 |
| 11 | 0,075 | 0,016 | 0,000 | 0,000 | 0,092 | 0,000 | 0,003 | 0,106 | 0,163 | 0,803 | 0,643 |
| 12 | 0,120 | 0,001 | 0,000 | 0,000 | 0,004 | 0,000 | 0,002 | 0,253 | 0,187 | 0,709 | 0,935 |
| 13 | 0,285 | 0,001 | 0,000 | 0,001 | 0,005 | 0,000 | 0,008 | 0,332 | 0,208 | 0,695 | 0,954 |
| 14 | 0,497 | 0,004 | 0,000 | 0,001 | 0,007 | 0,000 | 0,003 | 0,450 | 0,244 | 0,563 | 0,794 |
| 15 | 0,508 | 0,004 | 0,000 | 0,001 | 0,010 | 0,000 | 0,006 | 0,545 | 0,302 | 0,620 | 0,817 |
| 16 | 0,486 | 0,003 | 0,001 | 0,005 | 0,013 | 0,012 | 0,019 | 0,811 | 0,390 | 0,457 | 0,931 |
| 17 | 0,425 | 0,003 | 0,008 | 0,006 | 0,012 | 0,055 | 0,024 | 0,614 | 0,480 | 0,421 | 0,954 |
| 18 | 0,455 | 0,003 | 0,042 | 0,008 | 0,018 | 0,207 | 0,028 | 0,450 | 0,516 | 0,265 | 0,954 |
| 19 | 0,440 | 0,001 | 0,007 | 0,007 | 0,012 | 0,055 | 0,029 | 0,422 | 0,661 | 0,365 | 0,828 |
| 20 | 0,184 | 0,001 | 0,003 | 0,019 | 0,026 | 0,066 | 0,089 | 0,904 | 0,580 | 0,736 | 0,870 |
| 21 | 0,193 | 0,001 | 0,004 | 0,017 | 0,035 | 0,099 | 0,135 | 0,676 | 0,606 | 0,670 | 0,703 |
| 22 | 0,043 | 0,001 | 0,001 | 0,008 | 0,051 | 0,103 | 0,122 | 0,869 | 0,480 | 0,670 | 0,568 |
| 23 | 0,044 | 0,001 | 0,000 | 0,001 | 0,065 | 0,035 | 0,020 | 0,974 | 0,252 | 0,278 | 0,728 |
| 24 | 0,014 | 0,000 | 0,000 | 0,001 | 0,040 | 0,020 | 0,009 | 0,991 | 0,208 | 0,214 | 0,913 |
| 25 | 0,013 | 0,000 | 0,000 | 0,001 | 0,013 | 0,004 | 0,013 | 0,676 | 0,141 | 0,241 | 0,807 |
| 26 | 0,006 | 0,000 | 0,000 | 0,001 | 0,008 | 0,002 | 0,013 | 0,644 | 0,201 | 0,328 | 0,703 |
| 27 | 0,002 | 0,000 | 0,000 | 0,001 | 0,030 | 0,001 | 0,010 | 0,509 | 0,143 | 0,286 | 0,568 |
| 28 | 0,002 | 0,000 | 0,000 | 0,000 | 0,015 | 0,001 | 0,006 | 0,636 | 0,105 | 0,227 | 0,288 |
| 29 | 0,002 | 0,000 | 0,000 | 0,000 | 0,016 | 0,000 | 0,005 | 0,482 | 0,076 | 0,214 | 0,134 |
| 30 | 0,002 | 0,000 | 0,000 | 0,000 | 0,008 | 0,000 | 0,003 | 0,684 | 0,101 | 0,160 | 0,149 |
| 31 | 0,002 | 0,000 | 0,000 | 0,001 | 0,010 | 0,000 | 0,009 | 0,652 | 0,104 | 0,227 | 0,165 |
| 32 | 0,024 | 0,000 | 0,000 | 0,000 | 0,004 | 0,000 | 0,003 | 0,422 | 0,126 | 0,294 | 0,097 |
| 33 | 0,006 | 0,000 | 0,000 | 0,000 | 0,002 | 0,000 | 0,003 | 0,509 | 0,158 | 0,394 | 0,056 |
| 34 | 0,006 | 0,000 | 0,000 | 0,000 | 0,001 | 0,000 | 0,005 | 0,455 | 0,194 | 0,534 | 0,050 |
| 35 | 0,126 | 0,000 | 0,000 | 0,000 | 0,001 | 0,000 | 0,004 | 0,582 | 0,260 | 0,558 | 0,057 |
| 36 | 0,082 | 0,000 | 0,000 | 0,000 | 0,002 | 0,001 | 0,009 | 0,416 | 0,293 | 0,657 | 0,097 |
| 37 | 0,126 | 0,000 | 0,000 | 0,001 | 0,001 | 0,002 | 0,009 | 0,613 | 0,774 | 0,873 | 0,057 |
| 38 | 0,430 | 0,000 | 0,000 | 0,002 | 0,001 | 0,002 | 0,010 | 0,397 | 0,528 | 0,873 | 0,165 |
| 39 | 0,231 | 0,000 | 0,000 | 0,003 | 0,000 | 0,000 | 0,012 | 0,317 | 0,731 | 0,763 | 0,369 |
| 40 | 0,250 | 0,000 | 0,000 | 0,002 | 0,011 | 0,000 | 0,012 | 0,391 | 0,434 | 0,986 | 0,870 |
| 41 | 0,595 | 0,003 | 0,000 | 0,005 | 0,016 | 0,000 | 0,022 | 0,218 | 0,349 | 0,803 | 0,604 |
| 42 | 0,421 | 0,000 | 0,000 | 0,001 | 0,006 | 0,000 | 0,004 | 0,281 | 0,330 | 0,594 | 0,369 |
| 43 | 0,568 | 0,000 | 0,000 | 0,000 | 0,002 | 0,000 | 0,002 | 0,524 | 0,276 | 0,499 | 0,142 |
| 44 | 0,910 | 0,000 | 0,000 | 0,000 | 0,000 | 0,000 | 0,001 | 0,652 | 0,260 | 0,488 | 0,064 |
| 45 | 0,828 | 0,000 | 0,000 | 0,000 | 0,000 | 0,000 | 0,001 | 0,636 | 0,169 | 0,263 | 0,034 |
| 46 | 0,841 | 0,000 | 0,000 | 0,000 | 0,001 | 0,000 | 0,000 | 0,849 | 0,311 | 0,258 | 0,077 |
| 47 | 1,000 | 0,000 | 0,000 | 0,000 | 0,001 | 0,000 | 0,000 | 0,929 | 0,196 | 0,157 | 0,173 |
| 48 | 0,797 | 0,006 | 0,000 | 0,000 | 0,021 | 0,000 | 0,001 | 0,509 | 0,214 | 0,346 | 0,242 |
| 49 | 0,803 | 0,004 | 0,001 | 0,000 | 0,016 | 0,002 | 0,000 | 0,613 | 0,097 | 0,055 | 0,549 |
| 50 | 0,647 | 0,019 | 0,001 | 0,000 | 0,035 | 0,002 | 0,000 | 0,524 | 0,045 | 0,047 | 0,785 |
| 51 | 0,803 | 0,086 | 0,006 | 0,000 | 0,133 | 0,011 | 0,000 | 0,575 | 0,034 | 0,029 | 0,870 |
| 52 | 0,898 | 0,249 | 0,033 | 0,000 | 0,245 | 0,041 | 0,000 | 0,567 | 0,023 | 0,014 | 0,723 |
| 53 | 0,809 | 0,413 | 0,188 | 0,000 | 0,376 | 0,128 | 0,000 | 0,817 | 0,024 | 0,006 | 0,892 |
| 54 | 0,381 | 0,634 | 0,836 | 0,000 | 0,321 | 0,363 | 0,000 | 0,835 | 0,020 | 0,002 | 0,744 |
| 55 | 0,225 | 0,846 | 0,487 | 0,002 | 0,301 | 0,968 | 0,001 | 0,538 | 0,028 | 0,001 | 0,605 |
| 56 | 0,172 | 0,812 | 0,385 | 0,001 | 0,201 | 0,952 | 0,000 | 0,379 | 0,021 | 0,001 | 0,549 |
| 57 | 0,179 | 0,705 | 0,349 | 0,001 | 0,241 | 0,881 | 0,000 | 0,391 | 0,012 | 0,000 | 0,663 |
| 58 | 0,159 | 0,725 | 0,316 | 0,001 | 0,193 | 0,841 | 0,000 | 0,344 | 0,008 | 0,000 | 0,663 |
| 59 | 0,135 | 0,698 | 0,417 | 0,000 | 0,125 | 0,522 | 0,000 | 0,379 | 0,005 | 0,000 | 0,870 |
| 60 | 0,210 | 0,573 | 0,596 | 0,000 | 0,147 | 0,322 | 0,000 | 0,361 | 0,002 | 0,000 | 0,892 |
| 61 | 0,303 | 0,418 | 0,941 | 0,000 | 0,105 | 0,226 | 0,000 | 0,410 | 0,001 | 0,000 | 0,892 |

Statistically significant p-values (≤ 0.05) are highlighted in yellow

# 9 Diskussion

In dieser Arbeit wurde der Methylierungsstatus ausgewählter Gene ermittelt, um dessen Bedeutung für die Entstehung und Progression von myeloischen Neoplasien zu evaluieren. Ferner wurde überprüft, ob die aberrante Hypermethylierung der Gene einen prognostischen und diagnostischen Wert für diese Erkrankungen aufweist. Sechs Gene (*DAP-Kinase*, $p21^{CIP1}$, $p27^{KIP1}$, $p57^{KIP2}$, *SOCS-1,* $p15^{INK4B}$) wurden unter diesen Gesichtspunkten analysiert.

Eine *DAP-Kinase* Methylierung wurde in 47% (16/34) der untersuchten MDS Proben beschrieben[1]. Da eigene Voruntersuchungen mit einem quantitativen Assay diesem Bericht widersprachen, wurde eine größere Anzahl an Proben untersucht, um diese Unstimmigkeit zu klären. Es zeigte sich, dass die Methylierung des *DAP-Kinase* Gens im MDS ein eher unbedeutendes Ereignis darstellt und aufgrund der rein qualitativen Analyse stark überbewertet wurde[2] (Kapitel 2). Die detektierte Frequenz der Methylierung (43%, 31/73) stimmte zwar mit der beschriebenen Häufigkeit nahezu überein, ihr Level lag jedoch nur bei weniger als 5% und entsprach somit in etwa der Größenordnung die auch in normalem Gewebe gefunden wurde (0,5% - 2% in 9/20 Fällen). Auch eine Auswirkung auf die Expression durch diesen geringen Methylierungslevel konnte nicht festgestellt werden.

Vorhandene Berichte über *SOCS-1* Methylierung in AML[6,7] machten dieses Gen zu einem interessanten Untersuchungsziel in MDS. Die in AML beschriebene Hypermethylierung wurde in Exon 2 detektiert. Eine krankheitsspezifische Methylierung dieser Region konnte auch in einigen anderen Erkrankungen gezeigt werden[8-11]. Darüber hinaus war die Korrelation dieser Methylierung mit einer reduzierten Expression bekannt[12-16]. Aufgrund der Untersuchung einer anderen Region (Intron 1, irrtümlich als „Promotor“ oder „5′-UTR“ bezeichnet (Details im Anhang zu Kapitel 3) wurde die krankheitsspezifische Hypermethylierung des Gens in AML angezweifelt[17,18]. Um die Hypermethylierung von *SOCS-1* in MDS erstmalig zu untersuchen und ihre Relevanz in AML zu klären, wurde ein quantitativer Methylierungsassay für die Analyse von Exon 2 etabliert. Eine *SOCS-1* Hypermethylierung konnte in 31% (27/86) der untersuchten MDS- und in 48% (16/33) AML Proben detektiert werden[19] (Kapitel 3). Die Spezifität der Methylierungssignale wurde durch die Etablierung eines Schwellenwertes sichergestellt. Hierdurch wurde eine Fehlinterpretation schwacher Methylierungssignale (19 MDS, 3 AML) ausgeschlossen, welche auch in fast allen (20/24) gesunden Individuen gefunden wurden. Neben der

Entdeckung, dass die Hypermethylierung dieses Gens in MDS und AML ein häufiges Ereingis darstellt, konnte gezeigt werden, dass die Frequenz und der Level der Methylierung mit dem Blastengehalt der Erkrankung korrelieren. Darüber hinaus konnte in einem *in vitro* Experiment eine inverse Korrelation zwischen Hypermethylierung und Expression klar nachgewiesen werden. Zeitgleich wurden von einer anderen Arbeitsgruppe beide Regionen des *SOCS-1* Gens untersucht[20]. Diese Arbeit berichtet von einer seltenen „Promotor"-Methylierung in MDS (11%, 8/74), aber deren Abwesenheit in 56 AML- und 15 Kontrollproben. In Exon 2 wurde hingegen in 19/47 untersuchten AML-Fällen eine Hypermethylierung festgestellt. Der Methylierungsstatus von MDS in Exon 2 wurde nicht analysiert. Aufgrund einer in 2/15 Kontrollen gefundenen Methylierung im Exon 2 wurde geschlussfolgert, dass die Hypermethylierung dieser Region unspezifisch sei. Da diese Daten mit qualitativen Analysen generiert wurden, ist das Aufspüren von Signalen in den Kontrollproben allerdings kein Argument gegen die Relevanz von Methylierung in Exon 2. In der eigenen Arbeit wären bei einer rein qualitativen Auswertung sogar 20 von 24 Kontrollen als positiv bewerten worden. Dies verdeutlicht vielmehr die Notwendigkeit von quantitativen Analysen und der Festlegung von Schwellenwerten. Wie dieser Fall erneut zeigt, kommt es durch rein qualitative Untersuchungen leicht zu einer Überbewertung der unspezifischen Methylierungssignale, und deren Fehlinterpretation kann schnell zu einer falschen Schlussfolgerung führen. Die krankheitsspezifische Hypermethylierung von Exon 2 und deren biologische Auswirkung konnten durch die oben zitierten und die eigene Arbeit zweifelsfrei belegt werden. Darüber hinaus ist inzwischen widerlegt, dass, wie lange Zeit angenommen, eine relevante Methylierung nur in CpG-Inseln welche im 5´-Bereich der Gene lokalisiert sind, stattfinden kann. Es konnte nachgewiesen werden, dass auch GpG-Inseln, welche sich außerhalb der regulatorischen Bereiche befinden, in Neoplasien hypermethyliert sind und dass diese Hypermethylierung ebenfalls zu einem Expressionsverlust führen kann[21].

Eine Hypermethylierung der Zellzyklusinhibitoren der CIP/KIP Familie war in einigen hämatologischen Erkrankungen beschrieben, über den Methylierungszustand in MDS war jedoch wenig bekannt. In Bezug auf $p21^{CIP1}$, $p27^{KIP1}$ gab es keinerlei Informationen. Lediglich für $p57^{KIP2}$ war eine seltene Hypermethylierung (2/52 Fälle) beschrieben[3]. Die in derselben Arbeit beschriebene Abwesenheit von $p57^{KIP2}$ Methylierung in 14 untersuchten AML-Fällen stand im Widerspruch zu einer anderen Arbeit in welcher bei 7 von 25 untersuchten Fällen eine aberrante Hypermethylierung festgestellt wurde[4]. Diese

gegensätzliche Datenlage bei AML machte indirekt auch die Aussagekraft der Daten bezüglich MDS fraglich. Sämtliche Untersuchungen erfolgten rein qualitativ. Um den Methylierungsstatus dieser drei Gene in MDS und AML zu klären, wurden zuerst ebenfalls Untersuchungen mit qualitativen Assays durchgeführt[5] (Kapitel 4). Für $p27^{KIP1}$ konnte keine Methylierung nachgewiesen werden. Bei $p21^{CIP1}$ und $p57^{KIP2}$ hingegen wurden schwache Signale sowohl in den Patientenproben als auch in gesunden Individuen detektiert. Zur genaueren Untersuchung der Bedeutung dieser schwachen Signale wurden quantitative Assays entwickelt und die Proben erneut untersucht. Die Signale waren so schwach, dass sie mit dem stringenteren quantitativen Assay nicht erfasst werden konnten ($p57^{KIP2}$) oder sie lagen mit < 0,2% nur minimal über der Detektionsgrenze und wurden daher ebenfalls als negativ gewertet ($p21^{CIP1}$). Dies führte zu der Schlussfolgerung, dass die Methylierung der Gene der CIP/KIP Familie bei der Pathogenese von MDS und AML offensichtlich keine wesentliche Rolle zu spielen scheint.

Das in MDS am besten untersuchte Gen ist der Zellzyklusinhibitor $p15^{INK4B}$. Für dieses Gen ist eine starke intra- und interindividuelle Heterogenität des Methylierungsmusters in MDS und AML bekannt[22-24]. Derartige Methylierungsunterschiede sind mit den derzeit gängigen Methoden nicht zufriedenstellend erfassbar. Um detaillierte Informationen über den Methylierungszustand von $p15^{INK4B}$ in diesen Erkrankungen zu erhalten wurde eine weiterentwickelte Version der hochauflösenden Pyrosequenzierung verwendet (Kapitel 5). Hiermit wurden insgesamt 7762 einzelne CpG-Stellen in 82 Patientenproben und 32 Kontrollen untersucht. Mit dieser umfangreichen Analyse konnte gezeigt werden, dass es durch die Heterogenität innerhalb der CpG-Insel zu gravierenden regionalen Unterschieden bezüglich der Aussagekraft der detektierten Methylierung kommt. Es wurden Regionen mit sehr unterschiedlichem diskriminatorischen Potential identifiziert. Zwei Regionen zeigten signifikante Methylierungsunterschiede sowohl zwischen gesunden Individuen und Patientenproben, als auch zwischen den verschiedenen Erkrankungen. In anderen Bereichen hingegen unterschied sich die Methylierung kaum. Dies zeigt die dringende Notwendigkeit einer umfassenden und detaillierten quantitativen Analyse des Methylierungszustandes einer CpG-Insel. Bei heterogen methylierten Genen ist die Gefahr sehr hoch, dass signifikante Unterschiede, aufgrund einer Analyse von rein zufällig ausgewählten CpG-Stellen, nicht erkannt werden. Dies erhöht die Wahrscheinlichkeit einer falschen Bewertung des diagnostischen und prognostischen Wertes des untersuchten Gens und reduziert die Chance, dass potentielle Methylierungsmarker entdeckt werden.

Die Ergebnisse dieser Arbeit zeigen ausnahmslos, dass bei vielen Genen eine quantitative Methylierungsanalyse zwingend notwendig ist um die Spezifität der Signale zu gewährleisten. Da die Methylierungsunterschiede zwischen normalem Gewebe und Tumorgewebe häufig nicht qualitativer, sondern vor allem quantitativer Natur sind, kann es bei einer rein qualitativen Analyse zu einer deutlichen Überbewertung schwacher Methylierungssignale kommen. Dies führt durch deren Missinterpretation zu falsch-positiven Ergebnissen. Auch quantitative Unterschiede zwischen verschiedenen Stadien einer Erkrankung, welche ein großes prognostisches und diagnostisches Potential besitzen, lassen sich mit einer qualitativen Methode nicht erfassen. Bei Genen mit einer heterogenen Methylierung kommen erschwerend noch Unterschiede im Methylierungsmuster hinzu, welche ebenfalls eine entscheidende Bedeutung haben können (siehe Kapitel 5). In den Fällen, in denen sie vorhanden sind, müssen all diese Unterschiede mit hoher Sensitivität in einem großen Probenkollektiv analysiert werden. Nur dies garantiert eine spezifische Unterscheidung und ermöglicht die Identifizierung und Charakterisierung von potentiellen Methylierungsmarkern. Eine hierfür geeignete Methode muss also im Wesentlichen vier Kriterien erfüllen: 1. Quantifizierbarkeit, 2. Analyse vieler einzelner CpG-Stellen, 3. hohe Sensitivität und 4. Analyse einer großen Probenzahl. Die derzeit gängigen Methoden erfüllen weitestgehend nicht alle diese Kriterien. Aufgrund unterschiedlicher Vor- und Nachteile eignen sie sich für verschiedene Fragestellungen und können sich gegenseitig ergänzen. Ihre Vor- und Nachteile sollen nun diskutiert werden.

## *9.1 Vor- und Nachteile der geeignetsten gängigen Methoden*

Der momentane Goldstandard für eine detailierte Untersuchung des Methylierungsmusters ist das Sequenzieren von Bisulfit-behandelter DNA (*Bisulfit Genomic Sequencing*: BGS[25]). Diese Methode ermöglicht die Analyse vieler einzelner CpG-Stellen. Eine direkte Sequenzierung der Proben, welche auch eine semiquantitative Aussage zulässt, ist möglich, gestaltet sich jedoch in der praktischen Umsetzung meist schwierig und die Quantifizierung ist zudem sehr ungenau. Weiter verbreitet ist die Sequenzierung von Klonen. Hierbei mangelt es jedoch an der Sensitivität, denn die Detektion weniger methylierter Allele erfordert die Analyse einer sehr großen Anzahl von Klonen und ist somit extrem zeit- und arbeitsintensiv. Gleiches gilt entsprechend für die Untersuchung einer großen Probenzahl.

Bei der methylierungsspezifischen PCR (MSP)[26] handelt es sich um eine zeitsparende und kostengünstige Methode mit einer sehr hohen Sensitivität (ein Anteil von bis zu 0,1% methylierter DNA ist detektierbar). Diese Methode ermöglicht jedoch lediglich die Untersuchung einer Stichprobe der gesamten CpG Insel. Je nach Primerdesign werden etwa 3-6 CpG Stellen im Bereich der Primerbindungsstellen untersucht. Eine Auflösung der einzelnen CpG-Stellen ist hierbei nicht möglich, da nicht nachvollziehbar ist, wie viele und welche CpG-Stellen für das Binden der beiden Primer relevant sind. Ein weiterer, wesentlicher Nachteil ist die fehlende Möglichkeit der Quantifizierung. Dieses Problem ist relativ leicht zu lösen, indem man zusätzlich eine zwischen beiden Primern gelegene Sonde verwendet und eine quantitative MSP (MethyLight)[27] durchgeführt wird. Durch die Sonde kann, je nach Assaydesign, die Anzahl der untersuchten CpG-Stellen auf etwa 10 erhöht werden. Dies stellt allerdings immer noch einen relativ kleinen Anteil der gesamten CpG Insel dar, da diese durchaus einige hundert CpG-Stellen beinhalten kann. Darüber hinaus besteht auch weiterhin das Problem der fehlenden Einzel-CpG-Auflösung.

Die massenspektrometrische MALDI-TOF (Matrix Assisted Laser Desorption/Ionization – Time of Flight) Analyse hat als Hochdurchsatzverfahren den großen Vorteil, dass in kurzer Zeit viele CpG-Stellen in einer großen Anzahl von Proben untersucht werden können[28,29] Die Untersuchung einzelner CpG-Stellen ist mit dieser Methode jedoch häufig unmöglich, da keine geeigneten Fragmente generiert werden können, oder diese eine identische Länge aufweisen. Dies betrifft vor allem Bereiche mit einer hohen CpG-Dichte. Darüber hinaus ist die Zuverlässigkeit der Quantifizierung mittels MALDI-TOF-MS noch nicht abschließend geklärt.

Eine Array-basierte Methylierungsanalyse ermöglicht die gleichzeitige Analyse sehr vieler zu untersuchender Sequenzen in einer Probe[30,31] . Hierdurch eignet sich die Methode gut zur Untersuchung sehr vieler CpG-Inseln in wenigen Proben. Die Möglichkeit einzelne CpG-Stellen zu untersuchen ist stark abhängig von der Zusammensetzung der zu analysierenden Sequenz. Eine hohe CpG-Dichte und die geringe Sequenzkomplexität aufgrund der Natrium-Bisulfit-Behandlung (kaum Cs, viele Ts) erschwert die Untersuchung einzelner CpG-Dinukleotide. Selbst mit einem umfangreichen Assaydesign und sehr stringenten Filterkriterien war es bislang unmöglich, in diesen schwer zu untersuchenden Regionen CpG-Stellen einzeln zu analysieren[30,31].

Pyrosequenzierung stellt die derzeit vielversprechendste Methode für eine detaillierte Charakterisierung von Methylierungsmustern dar (siehe Kapitel 5). Diese Methode ermöglicht als einzige eine zuverlässige quantitative Analyse individueller CpG-Stellen. Es können mit vergleichsweise wenig Aufwand, in einem größeren Probenkollektiv, komplette CpG-Inseln detailliert charakterisiert werden. Die bisherige Limitierung der Methode bestand in der verfügbaren Software. Diese Software wurde ursprünglich für die Analyse von SNPs programmiert. Bei der Untersuchung mehrerer polymorpher Stellen wurden daher sämtliche Kombinationsmöglichkeiten errechnet. Aufgrund der hierfür benötigten Rechenleistung ist die Anzahl der mit einem Sequenzierprimer analysierbaren CpG-Stellen begrenzt und beschränkt sich in den meisten Studien auf wenige CpG-Stellen. Durch eine neue Methylierungssoftware, welche als beta-Version im Rahmen dieser Arbeit evaluiert werden konnte, wird die Anzahl analysierbarer Stellen deutlich erhöht. Mit dieser Software ist es nun möglich, mit einem Sequenzierprimer, bis zu 15 CpG-Stellen, welche über einen Bereich von knapp 100bp verteilt sind, zuverlässig zu charakterisieren. Hierdurch wird die Analyse einer kompletten CpG-Insel mit nur wenigen Sequenzierprimern ermöglicht.

Wie bereits erwähnt wurde, gibt es derzeit keine optimale Technik, sondern verschiedene Techniken ergänzen sich aufgrund unterschiedlicher Vor- und Nachteile sehr gut. Eine sinnvolle Strategie zur Identifizierung geeigneter Methylierungsmarker wäre die Kombination mehrerer Methoden. Hierfür sind verschiedene Kombinationsmöglichkeiten denkbar. Beispielsweise könnte zuerst mit Hochdurchsatzverfahren wie MALDI-TOF-MS eine grobe Voruntersuchung in einer sehr großen Probenzahl durchgeführt werden. Hiermit könnten viele Gene auf Methylierungsveränderungen in mehreren verschiedenen Geweben überprüft werden. Die CpG-Inseln der identifizierten Gene könnten anschließend mit einer sehr präzisen Methode, z.B. der Pyrosequenzierung, umfassend charakterisiert werden, um signifikante Unterschiede aufzuspüren, welche eine hohe Spezifität gewährleisten. Diese Unterschiede können dann mit sensitiven Methoden wie MSP (oder ML, sofern es ein quantitativer Unterschied ist) in größeren Probenzahlen erfasst werden. Bei sehr großen Probenzahlen (z.B. bei Routineuntersuchungen) könnte die Analyse, auch mehreren Marker gleichzeitig, mit Methylierungsarrays erfolgen.

## *9.2 Nutzen von Methylierungsmarkern*

Die derzeitige Diagnosestellung bei Krebserkrankungen erfolgt meist erst nach dem Erscheinen von Symptomen. Diese machen sich jedoch häufig erst relativ spät im Krankheitsverlauf bemerkbar. Aussagekräftigere Vorsorgeuntersuchungen könnten, durch eine frühzeitige Diagnose und einen rechtzeitigen Therapiebeginn, den Therapieerfolg und somit auch die Lebenserwartung deutlich erhöhen. Die Analyse von geeigneten Biomarkern könnte wesentlich zur Früherkennung und Risikoabschätzung beitragen. Veränderungen der DNA-Methylierung sind an der Entstehung und Progression vieler maligner Erkrankungen beteiligt und würden sich aus folgenden Gründen gut als Biomarker eignen:

1. In normalem Gewebe kommt Methylierung von CpG-Inseln nur relativ selten vor. Falls sie zu finden ist, so liegt ihr Level meist deutlich unter der Hypermethylierung welche in Tumorgewebe zu finden ist. Methylierungsunterschiede haben somit ein hohes diskriminatorisches Potential.

2. Aberrante Methylierung ist ein sehr frühes Ereignis während der malignen Veränderung von Zellen[32]. Sie konnte in einigen Tumoren, bereits Jahre vor der Krankheitsentstehung, in scheinbar normalen Epithelien und anderem Untersuchungsmaterial nachgewiesen werden[33-38] und eignet sich somit gut als Indikator zur Früherkennung und Risikoabschätzung[39]. Besonders bei Patienten mit einer erhöhten Prädisposition ist der hieraus resultierende präventive Nutzen offensichtlich.

3. Es gibt zum Teil deutliche Assoziationen zwischen quantitativen Veränderungen der Methylierung und dem Stadium einer Erkrankung (z.B. $p15^{INK4B}$ in MDS[24]; siehe Kapitel 5). Dies ermöglicht eine Subtypisierung ähnlicher Erkrankungen und hierdurch eine Verbesserung der Prognose (evtl. sogar der Therapie), sowie ein besseres Verständnis der Pathogenese dieser Erkrankungen.

4. Eine Verbesserung der Prädiktion wäre denkbar, sofern der Methylierungsmarker selbst für die phänotypischen Variation verantwortlich ist.

5. Die Verbreitung von Methylierungsmarkern ist vermutlich deutlich höher als die von genetischen Markern[40]. Methylierung von *GSTπ1* ist beispielsweise in über 90% Prostatakarzinomen[41,42] und *APC* Methylierung in über 90% in Ösophagus-Adenokarzinomen[43] zu finden.

6. Methylierung besitzt eine hohe biologische und chemische Stabilität und eignet sich dank dieser Beständigkeit gut als zuverlässiges und unkompliziertes Untersuchungsziel, auch aus Archivmaterial.

7. Eine Verwendung von Methylierungsunterschieden als Surrogatmarker wäre denkbar, wenn die Zusammenhänge zwischen Methylierung und reduzierter Expression bei einem Gen gut charakterisiert und zweifelsfrei geklärt sind. Die Veränderungen der Methylierung würden präzise Rückschlüsse auf den Expressionsstatus von mRNA und/oder Protein erlauben, welcher in vielen Fällen schwer bis unmöglich zu bestimmen sein kann. Dies gilt vor allem für Formalin fixiertes, in Paraffin eingebettetes Material.

8. Durch die detektierbare Dynamik der Methylierungsveränderungen wären eine Überwachung des Krankheitsverlaufes, sowie eine Kontrolle der Effizienz und des Verlaufes der Therapie möglich.

9. Es besteht die Möglichkeit einer Routineuntersuchung ohne größere invasive Eingriffe. Die Sensitivität vieler Methoden ist so hoch, dass die Detektion veränderter Methylierung in Patientenproben nicht auf Biopsien beschränkt ist, sondern auch für Blut und nahezu allen anderen Körperflüssigkeiten, -ausscheidungen und Lavagen erfolgreich gezeigt werden konnte[44,40].

Der enorme klinische Nutzen solcher Methylierungsmarker ist unbestritten. Der derzeitig limitierende Faktor sind vorwiegend die zurzeit verfügbaren Methoden. Trotz einiger Berichte über vielversprechende Methylierungsmarker[40] ist aufgrund unzureichender Sensitivität oder Spezifität ihres Nachweises, bisher kein solcher Marker etabliert worden. Diese bisherigen Aussagen beruhen allerdings überwiegend auf rein qualitativen Methylierungsanalysen mittels MSP. Wie in dieser Arbeit und anderen Veröffentlichungen[45-52] gezeigt wurde, kann die Spezifität durch exaktere Technologien deutlich erhöht werden. Durch die Festlegung genspezifischer Schwellenwerte können

falsch positive Ergebnisse nahezu ausgeschlossen werden. Eine Identifizierung signifikanter Bereiche ist durch hochauflösende Methoden möglich. Die Verwendung dieser Methoden und eine gleichzeitige Entwicklung von Methoden, welche eine optimale Identifizierung und Charakterisierung ermöglichen, würde die Aussicht auf die Etablierung eines zuverlässigen Methylierungsmarkers deutlich erhöhen. Darüber hinaus würde die Verwendung dieser Methoden generell zu einem verbesserten Verständnis der Rolle und Funktionen der DNA-Methylierung führen.

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# Publikationsliste

## *In dieser Dissertation enthaltene Publikationen*

1. U. Lehmann, **K. Brakensiek**, H. Kreipe:
   *Role of epigenetic changes in hematological malignancies.*
   Annals of Hematology, March 2004, Vol. 83, No. 3, pp. 137–152

2. **K. Brakensiek**, F. Langer, H. Kreipe, U. Lehmann:
   *Low level of DAP-kinase DNA methylation in myelodysplastic syndrome.*
   Blood, September 2004, Vol. 104, No. 5, pp. 1586-1587

3. **K. Brakensiek**, F. Langer, B. Schlegelberger, H. Kreipe, U. Lehmann:
   *Hypermethylation of the suppressor of cytokine signalling-1 (SOCS-1) in myelodysplastic syndrome.*
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4. **K. Brakensiek**, F. Langer, H. Kreipe, U. Lehmann:
   *Absence of $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$ methylation in MDS and AML.*
   Leukemia Research, November 2005, Vol. 29, No. 11, pp. 1357-1360

5. **K. Brakensiek**, L. U. Wingen, F. Langer, H. Kreipe, U. Lehmann:
   *Quantitative high resolution CpG island mapping using Pyrosequencing™ reveals disease-specific methylation patterns of the $p15^{INK4b}$ gene in Myelodysplastic Syndrome and Myeloid Leukaemia.*
   Clinical Chemistry (eingereicht)

## *Sonstige Arbeiten*

1. U. Lehmann, I. Berg-Ribbe, L. U. Wingen, **K. Brakensiek**, T. Becker, J. Klempnauer, B. Schlegelberger, H. Kreipe, P. Flemming:
   *Distinct methylation patterns of benign and malignant liver tumors revealed by quantitative methylation profiling.*
   Clinical Cancer Research, May 2005, Vol. 11, No. 10, pp. 3654-3660

2. O. Bock, J. Neuse, K. Hussein, **K. Brakensiek**, G. Büsche, T. Buhr, B. Wiese, H. Kreipe:
   *Aberrant collagenase expression in chronic idiopathic myelofibrosis (cIMF) is related to the stage of disease but not to the JAK2 mutation status.*
   American Journal of Pathology (im Druck)

3. K. Hussein, **K. Brakensiek**, M. Ballmaier, M. Bormann, G.Göhring, T. Buhr, O. Bock, H. Kreipe:
   *B-CLL developing in a patient with PV is not affected by V617F mutation of the Janus kinase 2.*
   Human Pathology (eingereicht)

## *Poster*

1. F. Länger, **K. Brakensiek**, J. Dingemann, H. Kreipe, U. Lehmann:
   *Heterogenität der DNA-Methyltransferase (DNMT)-Expression und Hypermethylierung im Myelodysplastischen Syndrom (MDS).*
   89. Jahrestagung der Deutschen Gesellschaft für Pathologie, 18.-21. Mai 2005, Wuppertal

2. O. Bock, K. Hussein, **K. Brakensiek**, G. Büsche, T. Buhr, H. Kreipe:
   *Detection of the single hotspot mutation in the pseudikinase domain JH2 of Janus Kinase 2 (JAK2) in bone marrow trephine biopsies derived from chronic myeloproliferative disorders.*
   3rd International Congress on Myeloproliferative diseases and Myelodysplastic syndromes, 27.-29.10.2005, Washington, DC, USA

3. U. Lehmann, **K. Brakensiek,** F. Länger, H. Kreipe:
   *DNA methylation of the tumor suppressor gene p15INK4b in myelodysplastic syndrome: High resolution quantitative DNA methylation profiling of a whole CpG island using pyrosequencing.*
   97th AACR Annual Meeting, 1.- 5. April 2006, Washington DC, USA

4. O. Bock, K. Hussein, **K. Brakensiek**, G. Büsche, T. Buhr, H. Kreipe:
   *Aktuelle Methoden zur Detektion der Punktmutation in der JH2 Pseudokinase Domäne der Janus Kinase 2 (JAK2) in Knochenmarkzellen von chronischen myeloproliferativen Erkrankungen.*
   90. Jahrestagung der Deutschen Gesellschaft für Pathologie, 19.-23. April 2006, Berlin

5. O. Bock, J. Neuse, K. Hussein, **K. Brakensiek**, G. Büsche, T. Buhr, B. Wiese, H. Kreipe:
   *Das Gen-Expressionsprofil von Kollagenen und Kollgenasen reflektiert den Verlauf der chronischen idioipathischen Myelofibrose.*
   90. Jahrestagung der Deutschen Gesellschaft für Pathologie, 19.-23. April 2006, Berlin

6. **K. Brakensiek,** F. Länger, H. Kreipe, U. Lehmann:
   *Umfassende, quantitative Methylierungsanalyse der CpG-Insel des Tumorsupressor-Gens p15INK4b mittels Pyrosequenzierung.*
   90. Jahrestagung der Deutschen Gesellschaft für Pathologie, 19.-23. April 2006, Berlin

## *Vorträge*

1. K. Hussein, **K. Brakensiek**, G. Büsche, T. Buhr, H. Kreipe, O. Bock:
   *Erhebung des Mutationsstatus der Janus Kinase 2 (JAK2) in isolierten Zellreihen von Philadelphia Chromosom negativen chronischen myeloproliferativen Erkrankungen.*
   90. Jahrestagung der Deutschen Gesellschaft für Pathologie, 19.-23. April 2006, Berlin

2. U. Lehmann, **K. Brakensiek**, K. Metzig, B. Hasemeier, P. Flemming, H. Kreipe:
   *DNA-Methylierungsmuster in Leberbiopsien von Hämochromatose-Patienten: Korrelation von Genotyp, Phänotyp und epigenetischen Veränderungen.*
   90. Jahrestagung der Deutschen Gesellschaft für Pathologie, 19.-23. April 2006, Berlin

# Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation selbstständig verfasst habe und die benutzten Hilfsmittel und Quellen, sowie evtl. zur Hilfeleistung herangezogene Institutionen vollständig angegeben wurden. Die Dissertation wurde nicht bereits als Diplom- oder ähnliche Prüfungsarbeit verwendet.

Hannover, den 03.05.2006

Kai Brakensiek