

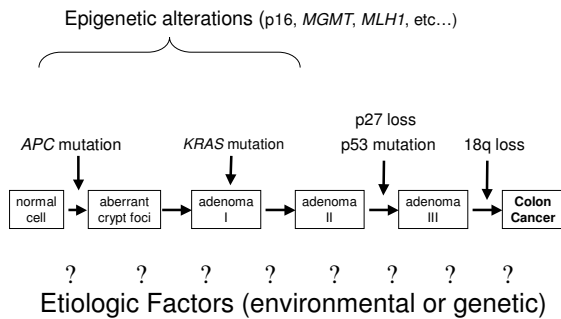
Clinical and Pathological Significance of Epigenomic Changes in Colorectal Cancer

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I have no conflict of interest

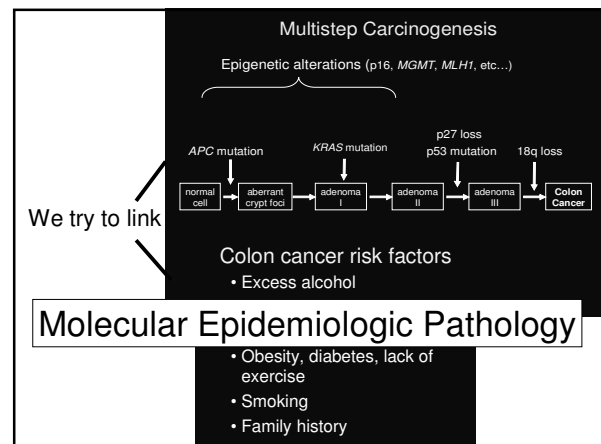
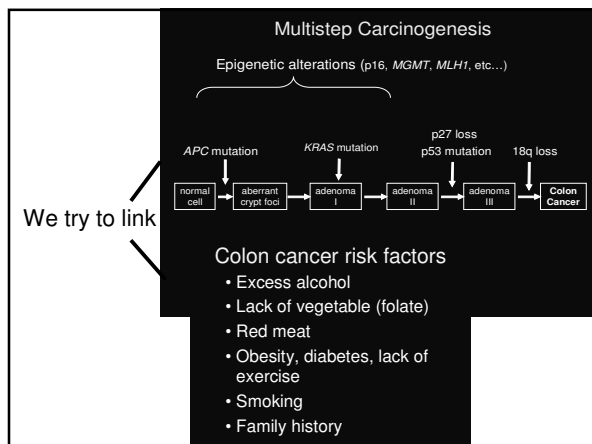
Brief overview of our epidemiology projects

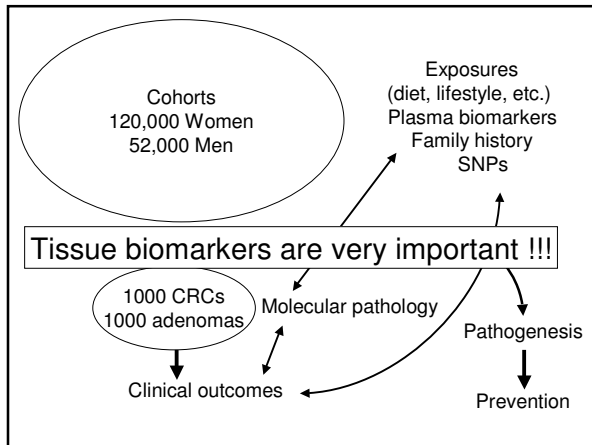
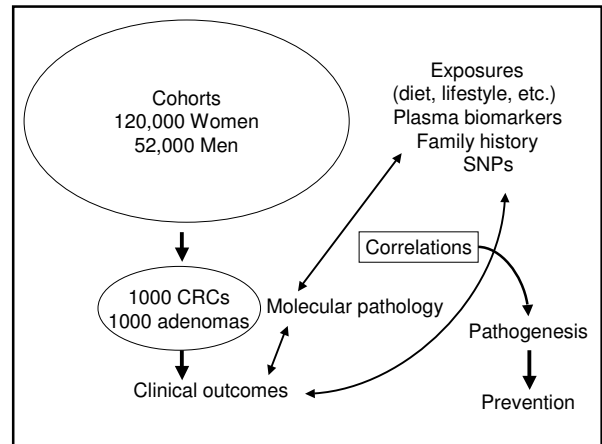
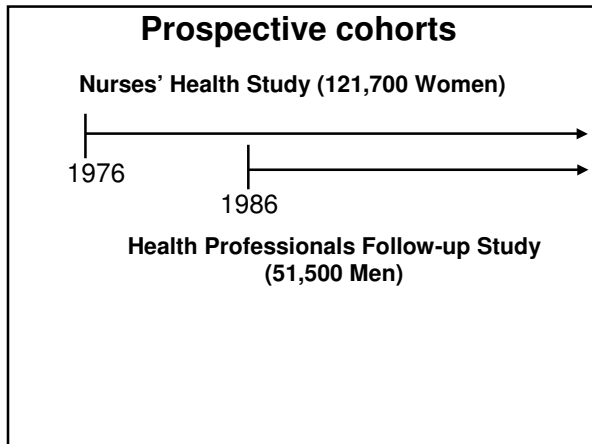
Multistep Carcinogenesis



Colon cancer risk factors

- Excess alcohol
 - Lack of vegetable (folate)
 - Red meat
 - Obesity, diabetes, lack of exercise
 - Smoking
 - Family history
- We have very limited knowledge about mechanisms



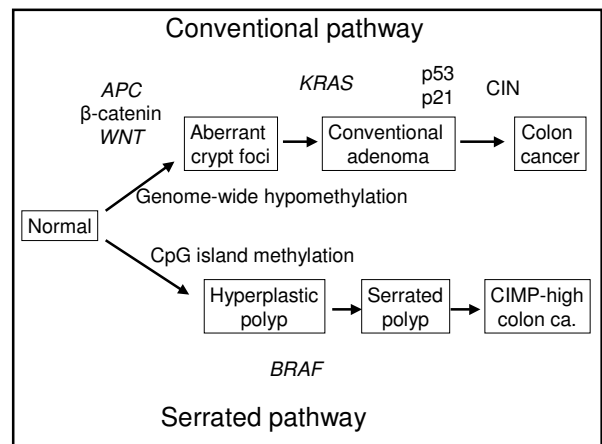


Epigenomic aberrations in colorectal cancer

Epigenomic aberrations

- Genome-wide DNA hypomethylation
 - Proto-oncogene activation
 - Genomic instability
- CpG island methylation
 - Tumor suppressor silencing

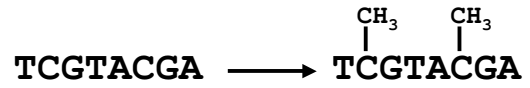
Mutually exclusive
Ogino et al. Int J Cancer 2008



Genome-wide DNA Hypomethylation

LINE-1

- LINE-1 (long interspersed nucleotide element-1) repetitive DNA elements have numerous CpG sites



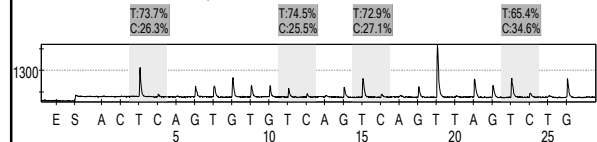
DNA methylation occurs at CpG (CG) sites

LINE-1

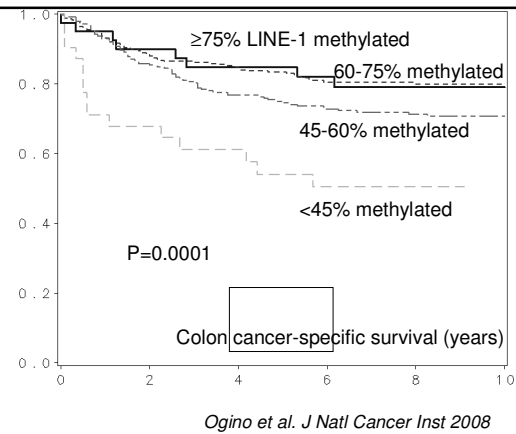
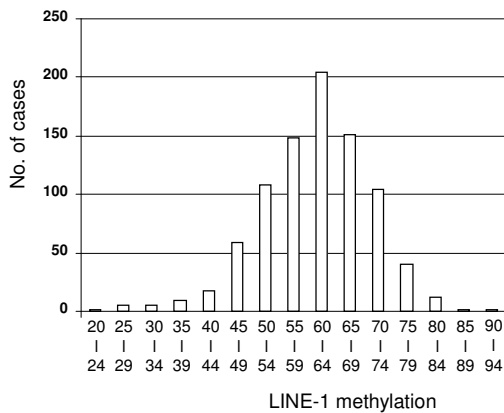
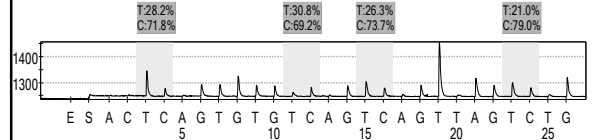
- LINE-1 methylation level correlates with genome-wide DNA methylation

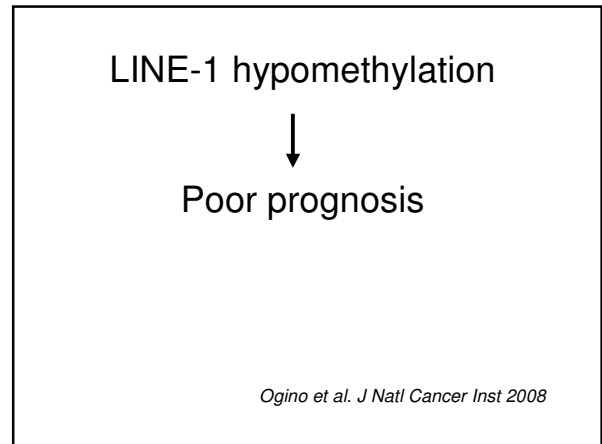
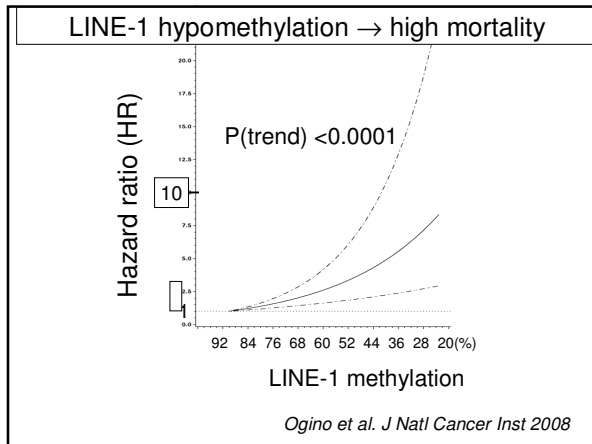
Pyrosequencing

27% LINE-1 methylation



73% LINE-1 methylation

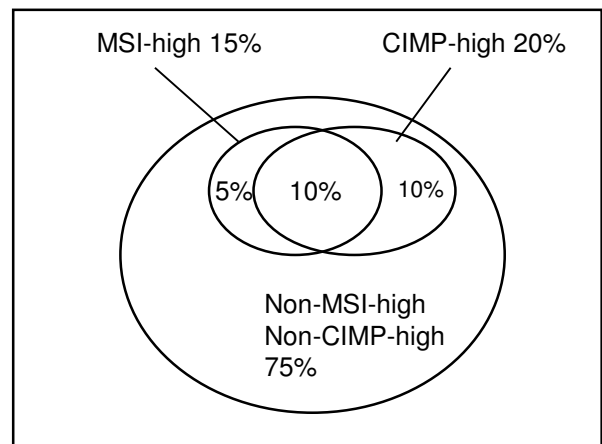




CpG island methylator phenotype (CIMP)

- CpG Island Methylator Phenotype (CIMP)
- Extensive promoter CpG island methylation
 - Inactivates many tumor suppressor genes
 - 20% of colorectal cancers show CIMP-high
 - Right colon, elderly female, *BRAF* mutation

- CIMP Causes Microsatellite Instability (MSI)
- MSI = altered lengths of microsatellites (short nucleotide repeats) in tumor
 - 15% of CRCs show MSI-high
 - 10% *MLH1* methylation in CIMP-high tumors
 - 2% Lynch syndrome (HNPCC)
 - 3% sporadic MSI-high, but not CIMP-high



Problems in CIMP Criteria

- Lack of validated consensus marker panel and criteria for CIMP-high

CIMP Panel Validation

- 900 colorectal cancers
- MethyLight (real-time PCR)
- 8-marker panel
 - *CACNA1G*, *CDKN2A* (p16), *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3*, *SOCS1* (including the new Laird's panel)

Ogino et al. J Mol Diagn 2007

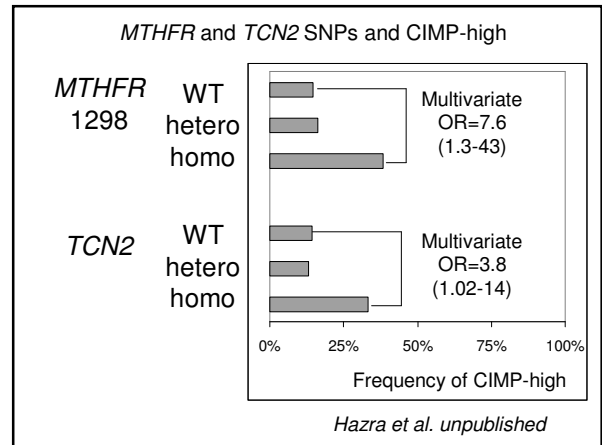
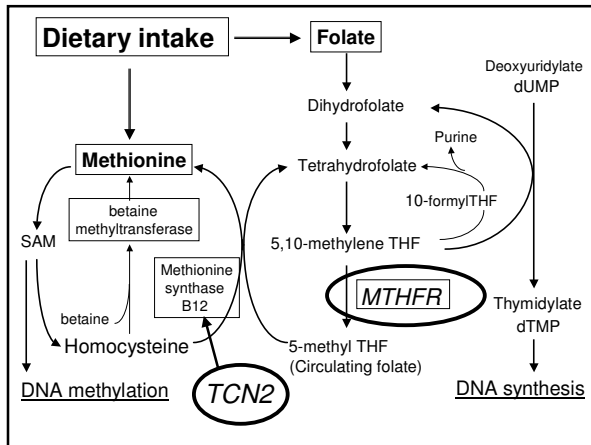
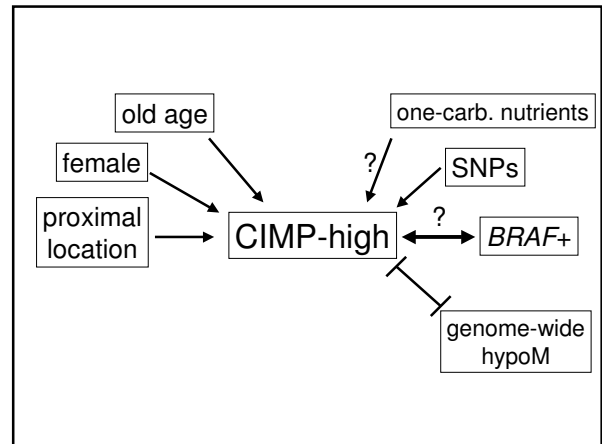
Unsupervised hierarchical clustering analysis of 16 methylation markers in 900 colorectal cancer

Nosho et al. PLoS ONE 2008

Nosho et al. PLoS ONE 2008

Now we have good methods, markers and criteria for CIMP-high

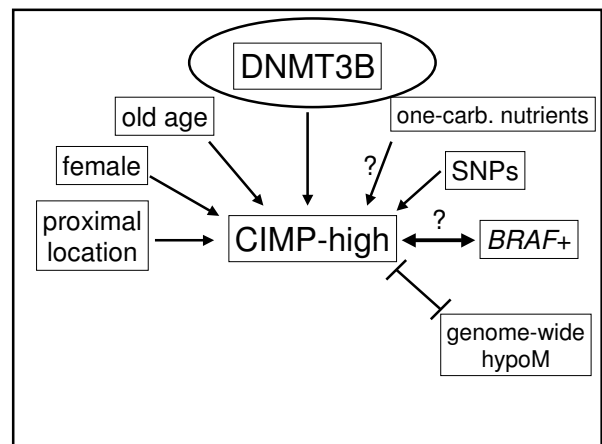
What causes CIMP-high?



Germline genetic variation



Somatic DNA methylation

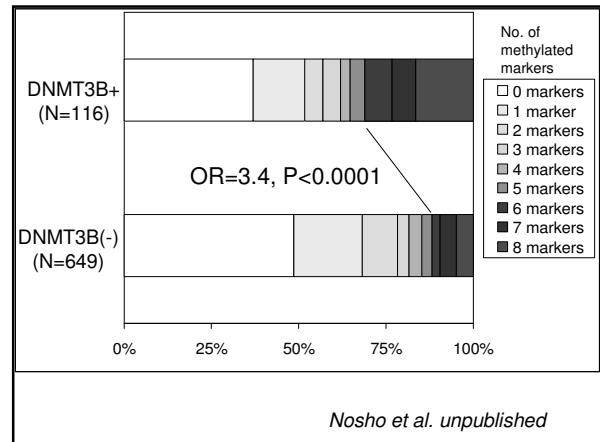


DNMT3B (DNA methyltransferase 3B)

Important enzyme for *de novo* DNA methylation

DNMT3B overexpression in mice induces CRC w CpG island methylation

Linhart et al. Gene Dev 2007



What causes CIMP-high?

Probably multifactorial

CIMP and *BRAF*

Clinical outcome

CIMP-high is good
(by the new CIMP panel)

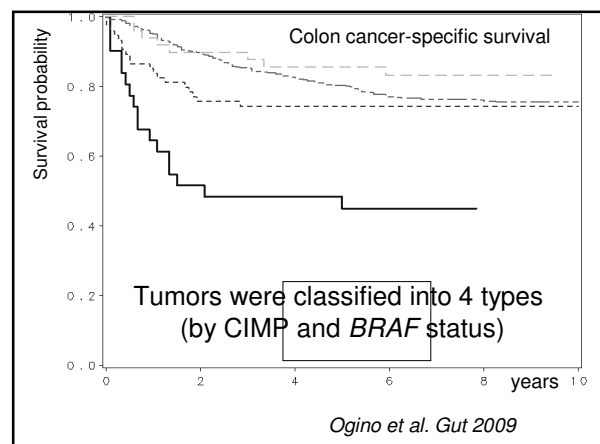


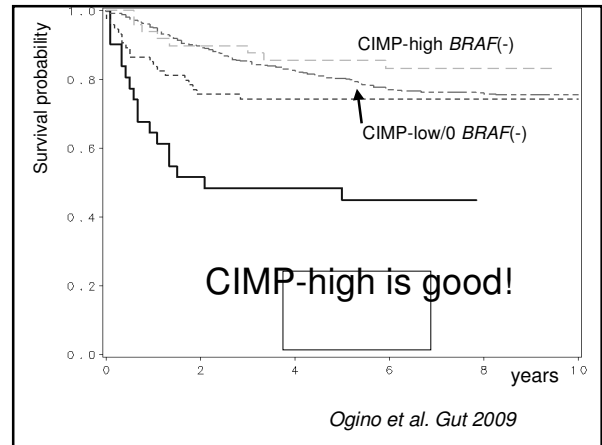
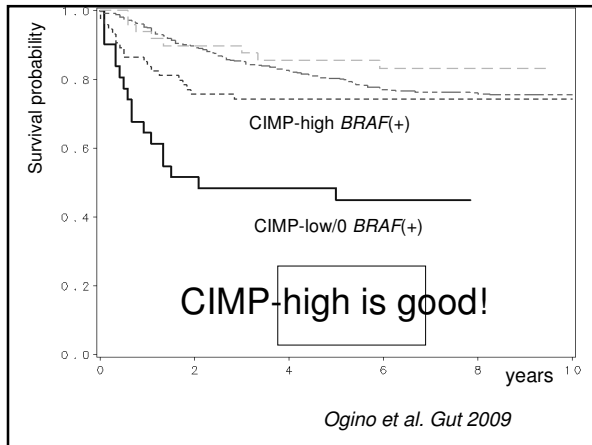
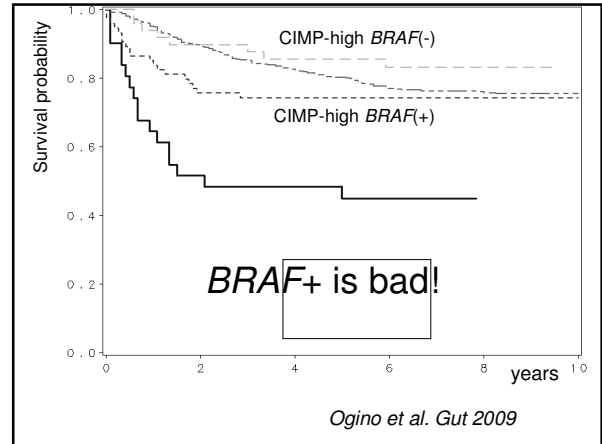
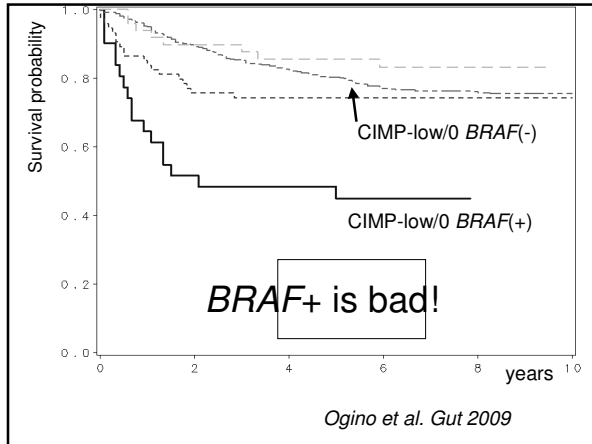
Independent



BRAF⁺ is bad

Ogino et al. Gut 2009





CIMP and BRAF are independent predictors

	Multivariate hazard ratio (95% CI)
CIMP-0	1
CIMP-low	0.8 (0.5-1.1)
CIMP-high	0.4 (0.2-0.9)
BRAF(-)	1
BRAF+	2.0 (1.1-3.4)

Ogino et al. Gut 2009

CIMP-low

CIMP-low (low-level CIMP)

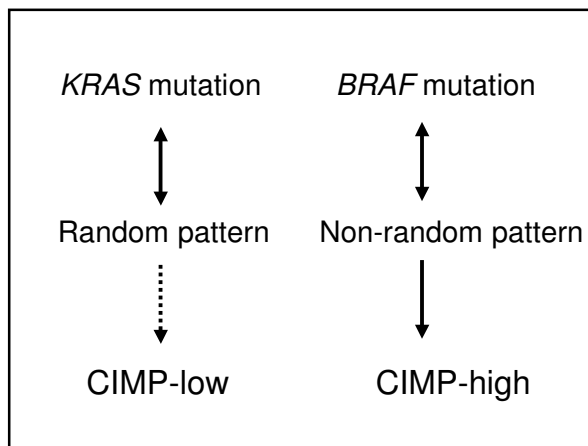
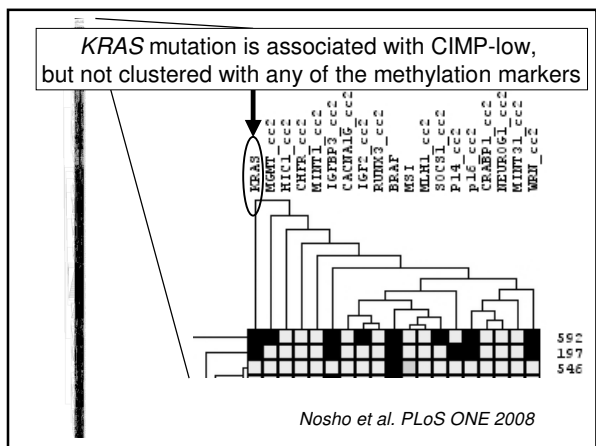
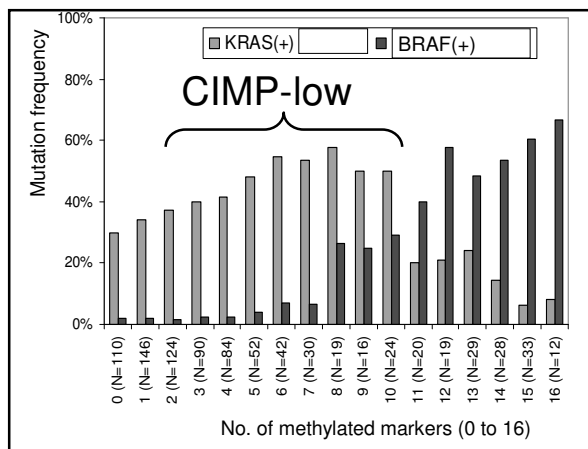
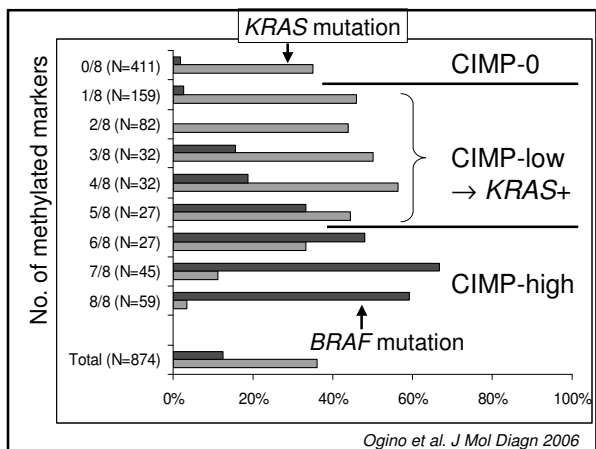
CpG island methylation is not as extensive as CIMP-high

CIMP-low

New epigenomic subtype ?

Ogino et al. J Mol Diagn 2006
Ogino et al. BMC Cancer 2007
Ogino et al. Gut 2007
Ogino et al. Mod Pathol 2008
Shima et al. unpublished

Or
 Mixture of CIMP-high and CIMP-0 ?
 Same as CIMP-neg (CIMP-0) ?



CIMP-high vs. CIMP-low vs. CIMP-0

<p><i>BRAF</i>⁺ Old age Female Proximal Inactive <i>WNT</i></p>		<p><i>KRAS</i>⁺ <i>KRAS/BRAF</i> WT Distal Genome-wide hypoM CIN</p>
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Summary 1

- CpG island methylator phenotype (CIMP-high)
 - Non-random methylation pattern (links to *BRAF* mutation)
 - inversely associated with genome-wide hypomethylation
 - DNMT3B may contribute to CIMP-high
- *KRAS*⁺ is associated with random methylation pattern (= CIMP-low)

Summary 2

CIMP-high → good prognosis

BRAF⁺ → bad prognosis

LINE-1 (i.e., genome-wide)
hypomethylation → bad prognosis

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Targeted Therapy of Melanoma: Kinases, Inhibitors and Molecular Correlates of Response

Dan Jones

University of Texas M.D. Anderson Cancer Center

Topics to be covered will be:

- The need for risk stratification and therapy response predictors in melanoma
- Pathways of transformation in melanoma
- Genetic status of the receptor tyrosine pathway (KIT, EGFR, BRAF and ras) and the use of kinase inhibitors
- Molecular diagnostic strategies to detect mutations in melanoma
- Cell cycle dysregulation and predictors of response to alkylating agents

Keywords: personalized medicine, targeted therapy, malignant melanoma, KIT, imatinib, temozolomide

Overview

Malignant melanoma represents one of the most aggressive malignancies but outcome is variable with early tumor lesions having an excellent prognosis following resection. Deregulated MAPK and AKT signaling by activating mutations in NRAS, BRAF, AKT, KIT and EGFR, or loss of PTEN represent molecular events leading to melanoma proliferation that are found at variable rates in different melanoma subtypes. Tumor suppressors involved in cell cycle function, DNA repair, and genome maintenance have a critical role in progression, with loss of p16 function and cyclin alterations being the most common. Profiling of mutations in growth signaling molecules and epigenetic changes associated with tumor progression may be identify the best candidates for targeted therapy.

I. Heterogeneity in melanoma: clinical presentation, and outcomes

Melanoma arises from malignant transformation of melanocytes and has an aggressive course once the tumor has spread from the superficial skin. However, like all tumor types there is considerable complexity and different approaches to subclassification. Patterns of melanoma related to risk, including acral lentiginous (AL), and the superficial spreading (SS), lentigo maligna (LM), and nodular types related to either severe and intermittent, or chronic sun exposure. Mucosal and soft tissue presentations of melanoma while rare appear to have a distinct pathogenesis. There are also a variety of different histologic appearances including typical epithelioid appearances, as well as desmoplastic (spindle cell) and anaplastic variants.

II. Outcomes and Therapies for melanoma

Outcome in melanoma is highly dependent on the depth of invasion seen in the primary lesion. Therefore, the predominant therapeutic modality in melanoma remains surgical resection with adequate margins. However, patients with residual disease after resection, or lymph node metastases or distant metastatic spread usually receive adjuvant therapy.

Particular groups of melanoma respond to immunotherapy (high-dose interferon with or without cell immunotherapy), or combination chemotherapy including an alkylating agents, usually dacarbazine (DTIC) or temozolomide (TMZ). Predictors of response to these adjuvant therapies are not yet clearly identified.

III. Melanoma: molecular activation of the growth signaling pathways and use of tyrosine kinase inhibitors

Almost all histologic and clinical patterns of melanoma are increased in patients with a history of heavy sun exposure, particularly discrete serious sunburn episodes but other risk factors are poorly understood. Approximately 10% of cases have strong familial link, and these cases have implicated cell cycle regulators, particularly p16 (CDKN2A) and CDK4, in molecular pathogenesis,^{1,2} which is discussed below. Most of the other known genetic alterations are found within commonly altered receptor tyrosine kinase (RTK) signaling pathways, which are particularly implicated in sun-exposed (cutaneous) cases.³ These genetic alterations include:

(a) **BRAF point mutation.** BRAF is a serine/threonine kinase downstream of RTKs and ras. 30-70% of melanomas show BRAF point mutations which alter the autoregulatory activation of the kinase, of which the V600E mutation is by far the most common. BRAF mutations are most common in the nodular and SS types, and rare in AL melanoma (5-10% of cases). However, benign nevi show similar or higher rates of the V600E BRAF mutation,⁴ and cell line and transgenic mouse models of melanoma do not clearly demonstrate the transforming power of this mutation. Germline BRAF mutations do not occur in melanoma.⁵

BRAF mutation correlates with distinct histopathologic features, such as intraepidermal melanoma nest formation and a larger rounder border of the tumor with surrounding skin, suggesting surrogate markers can be used to select cases for molecular testing. BRAF mutations arise more commonly in patients with younger age at presentation and lymph node metastasis (rather than satellite tumors or visceral metastasis).⁶

(b) **NRAS point mutation.** NRAS is a GTPase which functions to integrate signals from multiple RTKs. 10-20% of melanoma have point mutations in codon 12, 13 or 61 of NRAS. NRAS and BRAF mutations are almost always mutually exclusive,^{3,7} and ras-mutated melanoma seems to bypass BRAF and signal through CRAF.⁸ NRAS mutations are rarely found in benign acquired nevi (although seen in congenital nevi),⁹ arise later in melanoma development, can produce melanoma in certain animal models, and thus are more clearly implicated in oncogenesis than BRAF mutations.

(c) **KIT point mutations.** KIT is a RTK that is essential for normal neural crest/melanocyte differentiation. Activating KIT mutations are typically seen in mucosal and AL melanoma subtypes (20-40% of cases) but not in chronic sun damage cases, and include point mutations in KIT exons 11, 13 and 17.¹⁰ The L576P mutation is most common (comprising 50% of mutations). KIT gene amplification also occurs.^{4, 11} KIT mutations are mutually exclusive with BRAF and NRAS, and may identify a subset of melanoma that preferentially respond to the KIT inhibitors, imatinib (Gleevec),^{12, 13} or sorafenib.^{14, 15}

(d) **EGFR amplification.** EGFR is an RTK implicated in normal epithelial and melanocyte maturation. It is often overexpressed by gene amplification (usually whole gain of chromosome 7) in a majority of metastatic melanomas,¹⁶ but the diagnostic utility of gene amplification remains controversial. Rare cases of desmoplastic melanomas may show activating mutations.

(e) **PTEN gene deletion.** PTEN is a phosphatase that regulates the activation of the serine/threonine kinases AKT1/2/3, which are global regulators of cell proliferation. PTEN is regulated as a tumor suppressor with complete PTEN loss (usually by genomic deletion) seen in 20-25% of melanomas (including those with BRAF mutation),^{17, 18} and highly associated with uniform high-level activation of AKT in a melanoma. PTEN loss and concomitant AKT activation are both usually demonstrated by immunohistochemistry with anti-phosphoprotein antibody against an activation site on AKT (pS473).

(f) **AKT point mutations.** The AKT1 or rarely AKT3 isoform¹⁹ have been rarely shown to have activation mutations in sun-exposed melanoma subtypes, and AKT overexpression may be associated with melanoma growth in situ.^{20, 21}

(g) **FGFR2 mutations.** FGFR2 is a growth signaling cases that can mediate growth arrest in melanoma through interactions with stroma. Dominant-negative fibroblast growth factor (FGF) receptor mutations have been reported in approximately 10% of nodular melanomas.²²

Variations in the incidence of different mutations in different geographic populations are evident,²³ as well as variations in risk related to polymorphisms in other susceptibility loci.²⁴⁻²⁶

IV. Molecular diagnostic strategies for mutation detection

The strong association of particular growth pathway mutations with specific pathogenetic subtypes of melanoma arising at different tissue sites strongly supports microenvironmental influences on melanoma development. This implied dependency on supportive growth milieu also suggests that RTK and other kinase inhibitors may be successful in disrupting the trophic signals supporting melanoma,¹²⁻¹⁵ at least in early-stage lesions. Therefore, there is increasing clinical interest in RTK pathway mutational

profiling to help identify cases that may benefit from such KI treatment. Since there are now a number of genes with a variety of different sites mutated, several approaches to efficient profiling can be used.

(a) High-throughput strategies with definitive confirmation of identified mutations:

In this approach, DNA is extracted from all newly diagnosed tumors and many different genes (to include all those mentioned above) are interrogated using one of the high-throughput mutation discovery platforms (usually located in research core laboratories rather than clinical molecular laboratories). These methods could include the MassArray mass spectrometry method (Sequenom) or the pyrosequencing-based 454 method platform (Roche). Once a mutation is identified, it can then be confirmed in a (CLIA-compliant) setting in the clinical molecular diagnostics laboratory using a fully-validated method such as PCR-based Sanger sequencing..

(b) Probabilistic models to select which genes to test:

As mentioned above, mutations in melanoma are differentially observed depending on the site of presentation (AM versus chronic sun sites versus mucosal sites) and more weakly with histologic appearances (e.g. intraepidermal nesting of tumor cells in BRAF-mutated cases). Therefore, a tiered strategy for mutation detection based on these associations and the relevant frequency of mutations can be very successful.

For example, chronic sun-damage sites might be profiled for BRAF V600E, followed by NRAS exon 1/3, whereas mucosal and AL melanomas would be assessed for KIT L576P mutation, followed by KIT exons 13 and 17, or FGFR2, EGFR or AKT if histologic features warrant.

V. Which melanomas might benefit from chemotherapy with DNA-damaging agents

Although profiling of growth factor pathway alterations may be useful in selecting patients for KI therapy, NRAS and BRAF mutation status shows no correlation with response to chemotherapy or immunotherapy (there is as yet too limited data on cases with FGFR2, AKT or KIT mutations). The drugs typically used to treat melanoma include cis-platin, mitotic spindle poisons such as vinblastine, and alkylating agents. Resistance to cis-platin in melanoma may be related to sequestration in melanosomes,^{27, 28} and resistance to alkylating agents may be mediated by expression of the enzyme O(6)-methylguanine-DNA methyltransferase (MGMT) which opposes their action.²⁹ However, since therapeutic activity of alkylating agents and DNA-damaging agents require tumor cell division, melanomas with a higher proliferative rate or with genetic alterations in DNA maintenance pathways may be related to therapy response.³⁰

Like many tumor types, melanomas acquire several different genetic or epigenetic alterations in the genes that control the cell cycle checkpoint as tumors develop.³¹ p16 (CDKN2A) and p14 (CDKN2B) are inhibitors of the cyclin-dependent kinases (CDKs) which regulates the G1-S transition of the cell cycle. p16 and p14 act as a negative

regulators of the proliferation of normal cells by interacting strongly with CDK4 and CDK6 and inhibit their interaction with cyclin D. This blocks hyperphosphorylation of the retinoblastoma protein and the release of transcriptional activator E2A.

p14 and p16 are encoded in a complex multigene locus at chromosome 9p21 which is lost with tumor progression in many tumors, including melanoma. Loss of p14 or p16 function (often accompanied by upregulation of CDK4) leads to more rapid progression through the checkpoint, often leading to propagation of unrepaired DNA errors and subsequent mutagenic changes in tumor cells. Overexpression of cyclin D1 (CCND1) by gene amplification can also promote cell cycle progression, as seen in some melanomas.³² Loss of checkpoint function by mutation or deletion of TP53 (p53) is another promutagenic event leading to genetic instability.³³

As with growth factor gene alterations, different subtypes of melanoma show dramatic differences in the incidence of cell-cycle/checkpoint deficiencies, and the mechanisms by which they are altered (e.g. mutation, deletion or methylation silencing).³⁴ For example, melanomas arising in areas of sun-damage show frequent loss of TP53 (chromosome 17p14),³⁵ whereas p14 loss,³⁶ and CDK4 upregulation is more frequent in mucosal and acral lentiginous melanoma.³⁷ RTK pathway mutations, such as NRAS cooperate with p16/p14 loss in mouse models of melanoma.³⁸

Therefore profiling of the epigenetic and genetic alterations in cell cycle genes shows promise for more precisely selecting patients who might benefit from chemotherapy. Discussion of the best methods, given the variability ways that p14 and p16 may be silenced, has been published.³⁹

VII. Which melanomas might benefit from chemotherapy with spindle toxins

One of the most common classes of chemotherapeutic agents used for solid tumors is the spindle toxins, such as taxol and vincristine. These drugs block cell division by interfering with chromosomal segregation and cytokinesis. Vincristine and vinblastine are typically components of the multi-agent chemotherapy in melanoma.

These chemotherapeutic agents interfere with the progression of the mitotic spindle which is regulated by the spindle checkpoint complex that forms at the G2-M transition. This complex is a dynamic multi-protein structure that assures adequate centrosomal function and accurate chromosomal segregation. Proteins involved in the spindle checkpoint that are altered in cancer cells include cyclin B, the Aurora kinases, and RASSF1A. RASSF1A is a ubiquitously expressed gene which shows features of a tumor suppressor in many tumor types, including melanoma. There are at least 5 RASSF1A splice isoforms transcribed from different promoters that are differentially expressed. In melanoma, RASSF1C is ubiquitously expressed, but expression of the longer RASSF1A form is lost in 40% of metastatic melanoma by promoter CpG methylation silencing.

We have shown that loss of RASSF1A expression (by promoter methylation) in advanced stage melanoma is associated with response to chemotherapy and therefore may be a

biomarker of spindle dysfunction that synergizes with spindle toxins. Conversely, strategies to alter restore loss of spindle checkpoint proteins such as RASSF1A by use of demethylating agents may also be useful to limit ongoing genetic progression in patients with residual unresectable disease.

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Molecular Pathology of Acute Myeloid Leukemia: Prognostic and Predictive Genetic Tests

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The past 7 years has seen an explosion of knowledge about the molecular basis of acute myeloid leukemia (AML). An expanded menu of genetic tests is now available to assess prognosis and to predict response to specific therapy. The 2008 update of the WHO classification scheme for AML now categorizes about 2/3rds of cases by their genotype.¹ Guidelines for managing patients, such as the National Comprehensive Cancer Network (NCCN) guideline for AML, increasingly incorporate karyotype, fluorescence *in situ* hybridization (FISH), and reverse transcription polymerase chain reaction (rtPCR) technologies to monitor efficacy of treatment and to serve as early markers of relapse so that adjustments in therapy may be implemented before there is clinical evidence of recurrent disease.²

Prognostic Categories of Acute Myeloid Leukemia

Favorable risk factors

t(15;17)(q22;q12) *PML-RARA*

t(8;21)(q22;q22) *RUNX1-RUNX1T1*

inv(16)(p13;q22) or t(16;16)(p13;q22) *MYH11-CBFB*

NPM1 mutation without *FLT3* internal tandem duplication and with normal cytogenetics

CEBPA mutation (correlates with erythroid differentiation and higher hemoglobin)

RAS mutation

Intermediate risk group

Normal karyotype

FLT3 internal tandem duplication with *NPM1* mutation and normal cytogenetics

KIT mutation with t(8;21) or inv(16)

+8 only

t(9;11) *AF9-MLL* only

Unfavorable risk factors

Complex karyotype (≥ 3 abnormalities)

Monosomal karyotype (≥ 2 autosomal monosomies, or a single one plus ≥ 1 structural defect)

-5, -7, del(5q), del(7q) or other autosomal monosomy

11q23 *MLL* translocation, excluding t(9;11) *AF9-MLL*

MLL partial tandem duplication with normal cytogenetics

inv(3)(q21;q26) or t(3;3)(q21;q26) *RPN1-EVI1* or *MDS1/EVI1*

EVI1 overexpression

17p abnormality

FLT3 internal tandem duplication without *NPM1* mutation and with normal cytogenetics

t(9;22)(q34;q11) *BCR-ABL1*

t(6;9)(p23;q34) *DEK-CAN*

ERG overexpression without *FLT3* ITD and with *NPM1* mutation and normal cytogenetics

BAALC overexpression with normal cytogenetics

MN1 overexpression with normal cytogenetics

WT1 mutation with normal cytogenetics

Q-rtPCR in monitoring acute promyelocytic leukemia (APL)

Leukemia harboring t(15;17) *PML-RARA* uniquely responds high dose retinoic acid (all-trans-retinoic acid, ATRA), implying that is critical to identify leukemias having this genetic defect involving the retinoic acid receptor alpha (*RARA*) gene. Rare variant cases are found in which *RARA* has spliced with an alternate partner gene besides *PML*, and when *ZBTB16* (previously *PLZF*) or *STAT5B* is the partner then the tumor is likely to be resistant to ATRA.

Q-rtPCR can monitor tumor burden during therapy and rising levels can predict imminent relapse. After consolidation therapy, positive rtPCR test results are repeated to confirm them, and confirmed positivity is treated as if the patient had hematologic relapse. In contrast, patients having consistently negative rtPCR results, as tested at appx 2-3 month intervals, are likely to survive and may even be cured by current therapy.^{2,3} Testing should continue every 3 months for the next 2 years, and then every 6 months for 2-3 years. Any positive results, if confirmed on repeat, trigger treatment for relapse.

Genetic tests for prognosis of acute myeloid leukemia

Karyotype and molecular tests are key prognosticators in patients with AML.^{2,4-7} In cytogenetically normal AML, an assortment of informative molecular assays can be done, and the defects are not necessarily mutually exclusive. In fact, many tumors seem to harbor at least two genetic abnormalities, one that arrests differentiation and a second that promotes proliferation and survival. Some of the well known translocations may be insufficient for malignant transformation unless accompanied by a complementary defect(s).

Nucleotide-level defects in the *FLT3* and *NPM1* genes are among the most useful prognostic tests in cytogenetically normal AML. Patients with a poor prognosis are potential candidates for allogeneic stem cell transplantation.⁸

FLT3 internal tandem duplication confers a worse prognosis in cytogenetically normal AML. The relevant mutation is an in-frame internal tandem duplication (ITD) in the juxtamembrane domain of the *FMS-related tyrosine kinase 3* gene resulting in autophosphorylation and constitutive activation of the encoded *FLT3* tyrosine kinase. Signalling through the MAPK, PI3K and STAT5 pathways contributes to proliferation and cell survival. The mutation is typically detected by PCR followed by capillary electrophoresis to visualize an abnormally large (3 to 400 bp longer) amplicon associated with ITD.⁹ (See figure 4.) Some cancers lack a wild type allele and thus have a high ratio of mutant to wild type *FLT3* has been linked to an even worse prognosis.

NPM1 mutation confers a better prognosis in cytogenetically normal AML. *Nucleophosmin (NPM1)* mutation is quite prevalent and affected patients do very well when *FLT3* ITD is absent. On the other hand, they have an intermediate prognosis when *FLT3* ITD is present.¹⁰ The *NPM1* defect is insertion (or combined insertion and deletion) that usually has a net effect of increasing the size of exon 12 by 4bp and creating a frameshift.¹² The abnormal sequence encodes an *NPM1* nuclear shuttle protein that aberrantly localizes to the cytoplasm, adversely affecting its regulation of ARF-p53. "AML with a mutated *NPM1* gene" is now a provisional category of disease in the 2008 WHO classification system.¹ Several groups have designed assays to measure minimal residual disease based on sensitive quantification of the mutated *NPM1* DNA, but the utility of this approach was recently questioned because some relapsed tumors appear to have lost the mutation.¹³

Another new provisional WHO category is "AML with mutated *CEBPA*" which identifies a group of with favorable prognosis among patients with cytogenetically normal AML. *CEBPA* can be silenced by either mutation or by promoter hypermethylation, so mutation testing alone is inadequate to capture all of the relevant prognostic information. Interestingly, *CEBPA* dysfunction results in a higher hemoglobin level which has been proposed as a simple surrogate for *CEBPA* mutation or methylation testing.

When the *KIT* receptor tyrosine kinase gene is mutated in leukemias having t(8;21) or inv(16)) there is a worse clinical outcome. The capability of Gleevec or other tyrosine kinase inhibitors to overcome KIT activation is now being explored. *KIT* mutation is generally detected by sequencing exons 8 and 17 in leukemic cells.

RAS mutation is detected in about 15% of AMLs overall and in some subsets of leukemia it may confer a worse prognosis. Farnesyl transferase inhibitors (e.g. tipifarnib) have met with some success and predictive assays may help determine which patients are most likely to respond.¹⁹

Other prognostic factors in AML include abnormal expression of *ERG*, *BAALC*, *WT1*, *EVI1*, *MN1*, or *miR181*. In clinical settings, it is difficult to know how to manage the large numbers of prognostic factors in terms of clinical indications for testing each factor, and interpretation of results especially in light of our poor understanding of interactions among the various genetic and non-genetic factors. A predictive panel was proposed using *FLT3*, *NPM1*, *ERG*, *CEBPA* and *BAALC* genotypes to place normal karyotype patients into one of four groups with respect to the risks and benefits of stem cell transplantation.¹⁴ Arrays targeting either mRNA,¹⁵⁻¹⁸ micro RNA, or gene copy number are also being evaluated for their prognostic ability.

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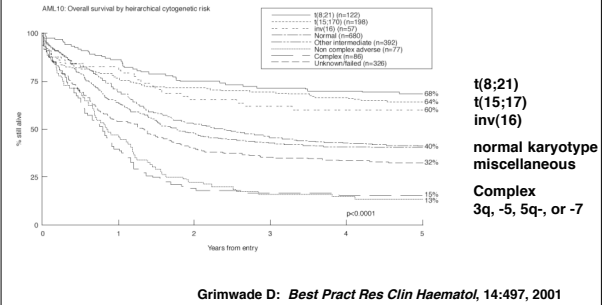
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Molecular Pathology of Acute Myeloid Leukemia: Prognostic and Predictive Genetic Tests

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March 8, 2009

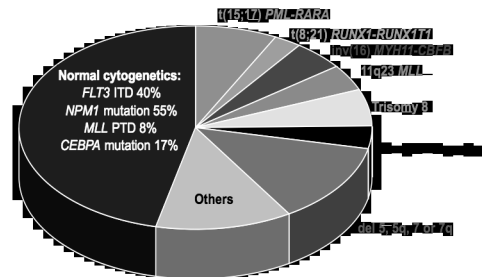
Genetics is the Major Prognostic Factor in AML



2008 WHO Classification of AML with Recurring Genetic Abnormalities

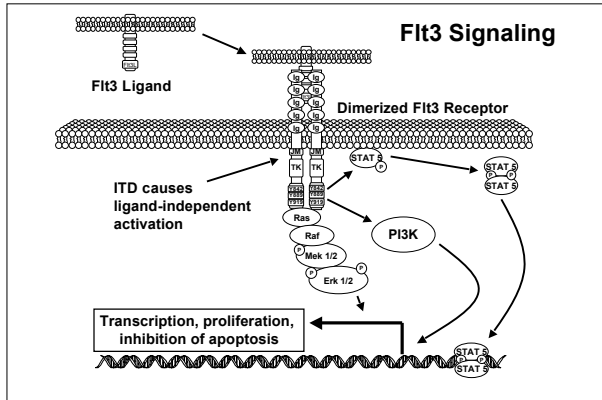
- AML with mutated *NPM1*
- AML with mutated *CEBPA*
- Myeloid and lymphoid neoplasms with eosinophilia and abnormalities of *PDGFRA*, *PDGFRB* and *FGFR1*
- AML with t(8;21)(q22;q22), *RUNX1-RUNX1T1*
- AML with inv(16)(p13;q22) or t(16;16)(p13;q22), *CBFB-MYH11*
- APL with t(15;17)(q22;q11-12), *PML-RARA*
- AML with t(9;11)(p22;q23), *MLL3-MLL*
- AML with t(6;9)(p23;q34), *DEK-NUP214*
- AML with inv(3)(q21;q26.2) or t(3;3)(q21;q26.2), *RPN1-EVI1*
- AML (megakaryoblastic) with t(1;22)(p13;q13), *RBM15-MKL1*
- Myeloid leukemia associated with Down syndrome

Relative Frequency of Common Genetic Abnormalities in Adult AML



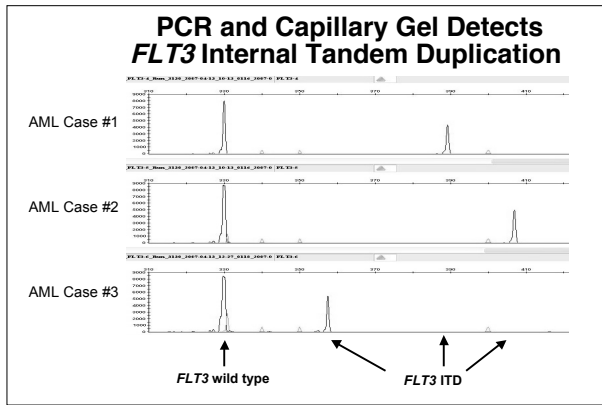
Favorable risk factors	Prognostic Genetic Characteristics in Acute Myeloid Leukemia
t(15;17)(q22;q12) <i>PML-RARA</i>	
t(8;21)(q22;q22) <i>RUNX1-RUNX1T1</i>	
inv(16)(p13;q22) or t(16;16)(p13;q22) <i>MYH11-CBFB</i>	
<i>NPM1</i> mutation without <i>FLT3</i> internal tandem duplication and with normal cytogenetics	
<i>CEBPA</i> mutation (correlates with erythroid differentiation and higher hemoglobin)	
<i>RAS</i> mutation	
Intermediate risk group	
Normal karyotype	
<i>FLT3</i> internal tandem duplication with <i>NPM1</i> mutation and normal cytogenetics	
<i>KIT</i> mutation with t(8;21) or inv(16)	
+8 only	
t(9;11) <i>AF9-MLL</i> only	
Unfavorable risk factors	
Complex karyotype (≥ 3 abnormalities)	
Monosomal karyotype (≥ 2 autosomal monosomies, or a single one plus ≥ 1 structural defect)	
-5, -7, del(5q), del(7q) or other autosomal monosomy	
11q23 <i>MLL</i> translocation, excluding t(9;11) <i>AF9-MLL</i>	
<i>MLL</i> partial tandem duplication with normal cytogenetics	
inv(3)(q21;q26) or t(3;3)(q21;q26) <i>RPN1-EVI1</i> or <i>MDS1/EVI1</i>	
<i>EVI1</i> overexpression	
17p abnormality	
<i>FLT3</i> internal tandem duplication without <i>NPM1</i> mutation and with normal cytogenetics	
t(9;22)(q34;q11) <i>BCR-ABL1</i>	
t(6;9)(p23;q34) <i>DEK-CAN</i>	
<i>ERG</i> overexpression without <i>FLT3</i> ITD and with <i>NPM1</i> mutation and normal cytogenetics	
<i>BAALC</i> overexpression with normal cytogenetics	
<i>MN1</i> overexpression with normal cytogenetics	
<i>WT1</i> mutation with normal cytogenetics	

Favorable risk factors	Prognostic Genetic Characteristics in Acute Myeloid Leukemia
t(15;17)(q22;q12) <i>PML-RARA</i>	
t(8;21)(q22;q22) <i>RUNX1-RUNX1T1</i>	
inv(16)(p13;q22) or t(16;16)(p13;q22) <i>MYH11-CBFB</i>	
<i>NPM1</i> mutation without <i>FLT3</i> internal tandem duplication and with normal cytogenetics	
<i>CEBPA</i> mutation (correlates with erythroid differentiation and higher hemoglobin)	
<i>RAS</i> mutation	
Intermediate risk group	
Normal karyotype	
<i>FLT3</i> internal tandem duplication with <i>NPM1</i> mutation and normal cytogenetics	
<i>KIT</i> mutation with t(8;21) or inv(16)	
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t(9;11) <i>AF9-MLL</i> only	
Unfavorable risk factors	
Complex karyotype (≥ 3 abnormalities)	
Monosomal karyotype (≥ 2 autosomal monosomies, or a single one plus ≥ 1 structural defect)	
-5, -7, del(5q), del(7q) or other autosomal monosomy	
11q23 <i>MLL</i> translocation, excluding t(9;11) <i>AF9-MLL</i>	
<i>MLL</i> partial tandem duplication with normal cytogenetics	
inv(3)(q21;q26) or t(3;3)(q21;q26) <i>RPN1-EVI1</i> or <i>MDS1/EVI1</i>	
<i>EVI1</i> overexpression	
17p abnormality	
<i>FLT3</i> internal tandem duplication without <i>NPM1</i> mutation and with normal cytogenetics	
t(9;22)(q34;q11) <i>BCR-ABL1</i>	
t(6;9)(p23;q34) <i>DEK-CAN</i>	
<i>ERG</i> overexpression without <i>FLT3</i> ITD and with <i>NPM1</i> mutation and normal cytogenetics	
<i>BAALC</i> overexpression with normal cytogenetics	
<i>MN1</i> overexpression with normal cytogenetics	
<i>WT1</i> mutation with normal cytogenetics	



FLT3 Internal Tandem Duplication (ITD) Assay

- **Specimen requirement:** EDTA blood or marrow with at least 10% leukemia cells
- **Method:** PCR across exons 14-15 followed by capillary gel electrophoresis
- **Analytic interpretation:** Enlarged amplicon (by 3 to 400 bp) implies ITD

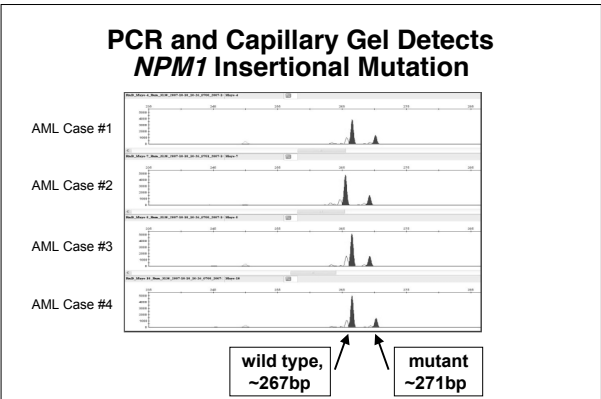


Clinical Utility of *FLT3* ITD Assay

- **Indication:** New AML with normal karyotype
- **Frequency:** Occurs in 25%
- **Clinical interpretation:** *FLT3* ITD implies a poor prognosis (consider allo transplant in first remission); high "ITD to wild type ratio" suggests even worse prognosis

NPM1 Mutation Assay

- Detects a ~ 4 base pair insertional mutation in exon 12 associated with aberrant cytoplasmic localization of the protein
- PCR across region followed by capillary gel electrophoresis



Clinical Utility of *NPM1* Mutation Assay

- **Indication:** New AML with normal karyotype
- **Frequency:** Half of AMLs have mutated *NPM1*
- **Clinical interpretation:** *NPM1* mutation implies a good prognosis; otherwise consider allo transplant in first remission

Monitoring minimal residual disease in acute myeloid leukaemia with *NPM1* mutations by quantitative PCR: clonal evolution is a limiting factor

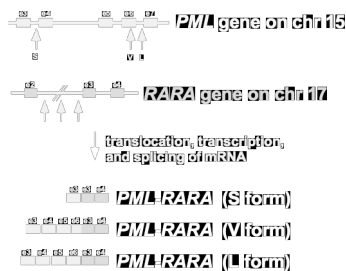
Christina Papadaki, Annika Dufour, Marlene Seibl, Stephanie Schneider, Stefan K. Bohlander, Evelyn Zellmeier, Gudrun Mellert, Wolfgang Hiddemann and Karsten Spiekermann
 Laboratory for Leukaemia Diagnostics and Department of Medicine III, University Hospital Grosshadern, Ludwig-Maximilians University, Munich, Germany

Summary

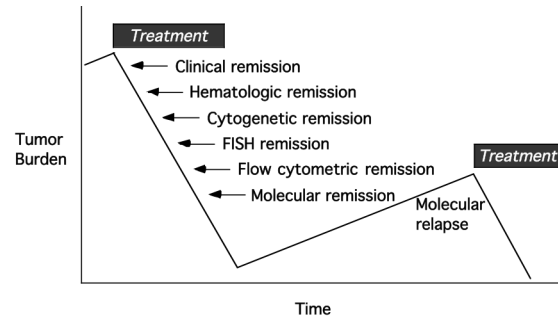
Nucleophosmin (*NPM1*) mutations in exon 12 represent the most frequent molecular aberrations in adult patients with acute myeloid leukaemia (AML). Molecular detection of *NPM1* mutation A could be a useful marker for routine monitoring of minimal residual disease (MRD). We established a calibrator-normalized relative quantification real-time polymerase chain reaction (PCR) assay for *NPM1* mutation A. *ABL1* was used as a reference housekeeping gene and the *NPM1* mutation A-containing OCI/AML3 cell line as a calibrator. Relative quantification was performed by calculating the *NPM1* mutation A/*ABL1* ratio which was normalized to the *NPM1* mutation A/*ABL1* ratio of OCI/AML3 calibrator cDNA. The assay showed a sensitivity of 10^{-5} . The clinical usefulness was evaluated by monitoring MRD in 51 AML

Br J Haematol 2008 Nov 26. PMID: 19055671

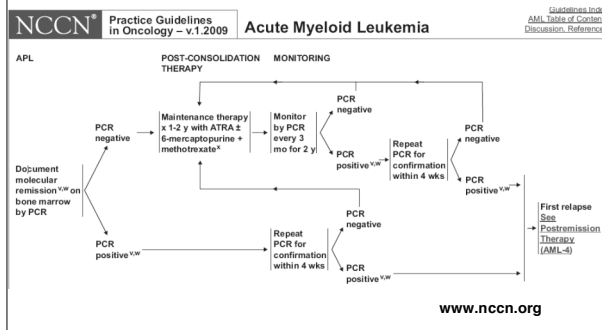
Molecular Anatomy of t(15;17) *PML-RARA*



Tumor Burden Measures Efficacy of Therapy



Practice Guidelines for rtPCR of *PML-RARA*



Heritable Syndromes Predisposing to AML

Syndrome	Gene symbol	Locus
Bloom syndrome	<i>BLM</i>	15q26.1
Li-Fraumeni syndrome	<i>TP53</i>	17p13
Down syndrome	-	trisomy 21
Ataxia telangiectasia	<i>ATM</i>	11q22.3
Fanconi anemia A	<i>FANCA</i>	16q24.3
Fanconi anemia C	<i>FANCC</i>	9q22.3
Fanconi anemia D2	<i>FANCD2</i>	3p26
Fanconi anemia E	<i>FANCE</i>	6p21-p22
Fanconi anemia F	<i>FANCF</i>	11p15
Fanconi anemia G	<i>FANCG</i>	9p13
Fanconi anemia J	<i>BRIP1</i>	17q22
Fanconi anemia N	<i>PALB2</i>	16p12.1
Schwachman-Diamond syndrome	<i>SBDS</i>	7q11

Reliable websites on Cancer Genetics

<http://atlasgeneticsoncology.org>

<http://cgap.nci.nih.gov/chromosomes/Mitelman>

www.ncbi.nlm.nih.gov/Literature/index.html

www.ncbi.nlm.nih.gov/sites/entrez?db=cancerchromosomes

www.amp.org

Favorable risk factors	Prognostic Genetic Characteristics in Acute Myeloid Leukemia
t(15;17)(q22;q12) <i>PML-RARA</i>	<p>Prognostic Genetic Characteristics in Acute Myeloid Leukemia</p> <p><i>t(8;21)(q22;q22) RUNX1-RUNX1T1</i> <i>inv(16)(p13;q22) or t(16;16)(p13;q22) MYH11-CBFB</i> <i>NPM1</i> mutation without <i>FLT3</i> internal tandem duplication and with normal cytogenetics <i>CEBPA</i> mutation (correlates with erythroid differentiation and higher hemoglobin) <i>RAS</i> mutation</p> <p>Intermediate risk group Normal karyotype <i>FLT3</i> internal tandem duplication with <i>NPM1</i> mutation and normal cytogenetics <i>KIT</i> mutation with t(8;21) or inv(16)</p> <p>Unfavorable risk factors Complex karyotype (≥ 3 abnormalities) Monosomal karyotype (≥ 2 autosomal monosomies, or a single one plus ≥ 1 structural defect) -5, -7, del(5q), del(7q) or other autosomal monosomy 1q23 <i>MLL</i> translocation, excluding t(9;11) <i>AF9-MLL</i> <i>MLL</i> partial tandem duplication with normal cytogenetics inv(3)(q21;q26) or t(3;3)(q21;q26) <i>RPN1-EV11</i> or <i>MDS1/EV11</i> <i>EV11</i> overexpression 17p abnormality <i>FLT3</i> internal tandem duplication without <i>NPM1</i> mutation and with normal cytogenetics t(9;22)(q34;q11) <i>BCR-ABL1</i> t(6;9)(p23;q34) <i>DEK-CAN</i> <i>ERG</i> overexpression without <i>FLT3</i> ITD and with <i>NPM1</i> mutation and normal cytogenetics <i>BAALC</i> overexpression with normal cytogenetics <i>MN1</i> overexpression with normal cytogenetics <i>WT1</i> mutation with normal cytogenetics</p>
t(8;21)(q22;q22) <i>RUNX1-RUNX1T1</i>	
inv(16)(p13;q22) or t(16;16)(p13;q22) <i>MYH11-CBFB</i>	
<i>NPM1</i> mutation without <i>FLT3</i> internal tandem duplication and with normal cytogenetics	
<i>CEBPA</i> mutation (correlates with erythroid differentiation and higher hemoglobin)	
<i>RAS</i> mutation	
Intermediate risk group	
Normal karyotype	
<i>FLT3</i> internal tandem duplication with <i>NPM1</i> mutation and normal cytogenetics	
<i>KIT</i> mutation with t(8;21) or inv(16)	
t(9;11) <i>AF9-MLL</i> only	
Unfavorable risk factors	
Complex karyotype (≥ 3 abnormalities)	
Monosomal karyotype (≥ 2 autosomal monosomies, or a single one plus ≥ 1 structural defect)	
-5, -7, del(5q), del(7q) or other autosomal monosomy	
1q23 <i>MLL</i> translocation, excluding t(9;11) <i>AF9-MLL</i>	
<i>MLL</i> partial tandem duplication with normal cytogenetics	
inv(3)(q21;q26) or t(3;3)(q21;q26) <i>RPN1-EV11</i> or <i>MDS1/EV11</i>	
<i>EV11</i> overexpression	
17p abnormality	
<i>FLT3</i> internal tandem duplication without <i>NPM1</i> mutation and with normal cytogenetics	
t(9;22)(q34;q11) <i>BCR-ABL1</i>	
t(6;9)(p23;q34) <i>DEK-CAN</i>	
<i>ERG</i> overexpression without <i>FLT3</i> ITD and with <i>NPM1</i> mutation and normal cytogenetics	
<i>BAALC</i> overexpression with normal cytogenetics	
<i>MN1</i> overexpression with normal cytogenetics	
<i>WT1</i> mutation with normal cytogenetics	

Position of Activating Mutation in *KIT* May Correlate with TKI Drug Response*

Activating Mutation (AA)	<i>KIT</i> Exon	Gleevec Response
dup 502+503	9	?
dup 577-583	11	Yes
del 554-559	11	Yes
W557R	11	?
K558N	11	Yes
V559D	11	Yes
L576P	11	Yes
V553N	11	?
V559A	11	Yes
N566D	11	?
V569G	11	?
R634W	13	?
K642E	13	Yes
D816H	17	Resistant
Y823D	17	Resistant
T823D	17	Resistant
A829P	17	Resistant

* must be validated for each disease category

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Intermediate risk group	
Normal karyotype	
<i>FLT3</i> internal tandem duplication with <i>NPM1</i> mutation and normal cytogenetics	
<i>KIT</i> mutation with t(8;21) or inv(16)	
+8 only	
t(9;11) <i>AF9-MLL</i> only	
Unfavorable risk factors	
Complex karyotype (≥ 3 abnormalities)	
Monosomal karyotype (≥ 2 autosomal monosomies, or a single one plus ≥ 1 structural defect)	
-5, -7, del(5q), del(7q) or other autosomal monosomy	
1q23 <i>MLL</i> translocation, excluding t(9;11) <i>AF9-MLL</i>	
<i>MLL</i> partial tandem duplication with normal cytogenetics	
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t(9;22)(q34;q11) <i>BCR-ABL1</i>	
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<i>BAALC</i> overexpression with normal cytogenetics	
<i>MN1</i> overexpression with normal cytogenetics	
<i>WT1</i> mutation with normal cytogenetics	

Multiple stand-alone reports
or comprehensive report synthesizing
multiple tests done on the specimen

- Morphology (pathologist "consultation")
- Immunophenotype (flow, IHC)
- Karyotype
- Molecular (FISH, ISH, PCR, sequencing, etc)
- Array (pathologist "consultation")

The Pathologist is a Physician Consultant

"Because of the multidisciplinary approach required to diagnose and classify myeloid neoplasms it is recommended that the various diagnostic studies be correlated with the clinical findings and reported in a single, integrated report."

Vardiman et al, WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues (IARC, 2008)