

what Molecular targets

- Rearrangements
 - physiologic
 - pathologic
- Mutations
- Additions
- Losses
 - deletions
 - silencing

why Major Indications

- **diagnosis**
 - neoplastic vs reactive (and beyond)
- **classification**
 - based upon the genetic lesion
- **prognosis**
 - in otherwise homogeneous diseases
- **monitoring/MRD**
 - both response and early recurrence

how Major methodologies

Cytogenetics

FISH CGH SKY

Molecular genetics

PCR

Advantages of molecular methods

	Karyotypic	Molecular
Fresh material	Yes	No
Viable cells	Yes	No
Dividing cells	Yes	No
Average TAT	~1 week	~ 2 days
Submicroscopic	No	Yes
Numeric	Yes	No
Sensitivity	5-10%	0.001-1%

Why and how to go FISHing

GO FISH!

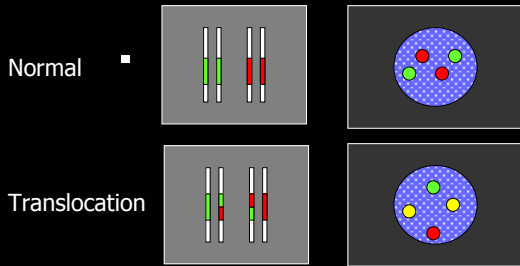
why

- no need for:
 - metaphases
 - fresh cells
 - proliferating cells

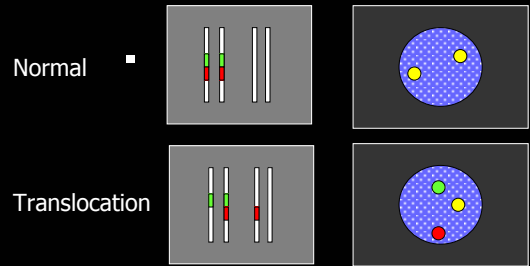
how

- Fusion probes
- Split-apart probes
- Centromeric probes

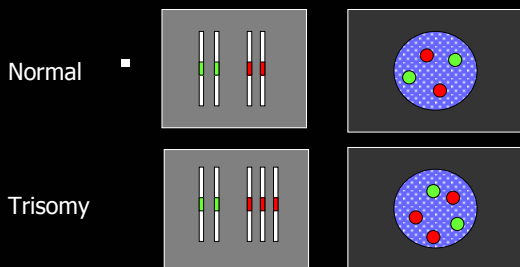
How to go FISHing: fusion probes



How to go FISHing: split part probes



How to go FISHing: centromeric probes

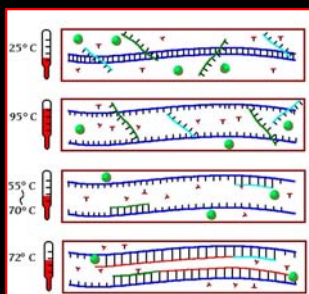


Why Southern blotting has gone south

Advantages of PCR-based testing

- Automation
- Faster
- DNA or RNA
- Minimal tissue
- Fixed tissue
- Sensitivity
- Multiplexing
- No hot stuff
- Cost effective

A pictorial PCR primer



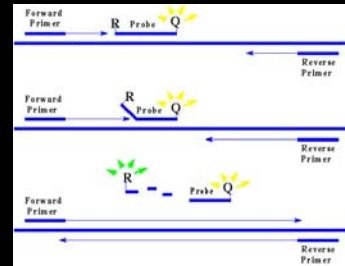
Flavors of PCR

- Regular - DNA
- RT-PCR - RNA
- Multiplex-PCR - single target gene: *TCRG*; translocation: *t(15;17)*
multiple targets numerous translocations
- Nested PCR - same product- internal primers
- Quantitative PCR - competitive
- limiting dilution
- real time

Flavors of Real time PCR

- Single probe
 - Taqman
 - Molecular beacon
- Two probes
 - FRET
- No probe
 - intercalating dye
 - * SYBR green
 - * melting curves

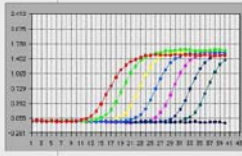
Real time PCR: Taqman probe



Real time PCR: amplification curves

Real-Time PCR detection

The automatic detection of PCR product growth throughout the amplification process.



- Collects geometric phase data
- No post-PCR steps
- Enables high precision and high throughput

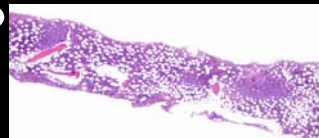
The toolbox and the tasks ...

- Rearrangements (qualitative and quantitative)
 - physiologic
 - pathologic
 - PCR: homogeneity versus heterogeneity
 - PCR and FISH: presence or absence
- Mutations
 - gold standard
 - others
 - screening
 - Sequencing
 - Restriction endonucleases
 - ASO (allele specific oligonucleotides)
 - SSCP, DGGE, TGGE
- In situ hybridization
 - morphologic correlate
 - EBV (EBERs), Ig κ and λ
- Other clonality
 - XCIP
 - HUMARA

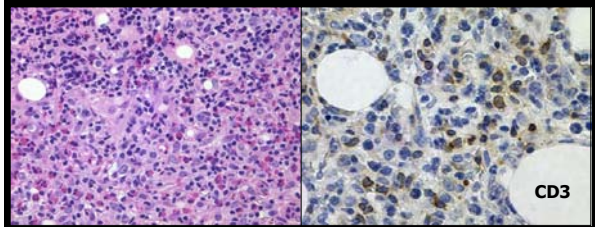
CASE 1

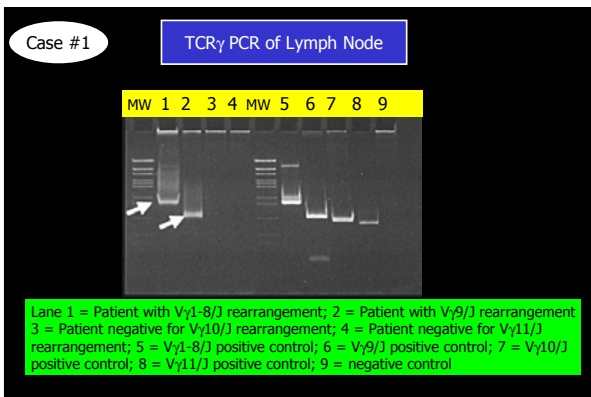
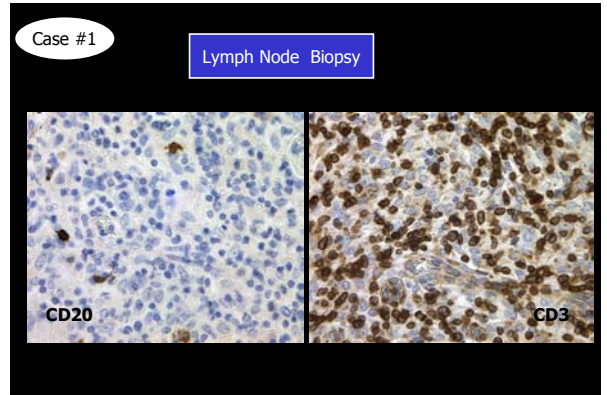
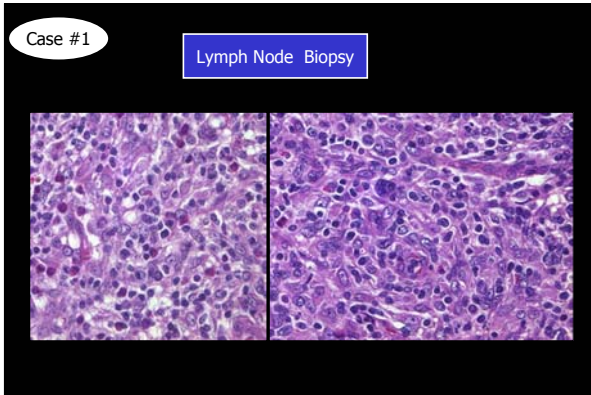
60-year-old man with a history of fever and night sweats was found to have lymphadenopathy, normocytic anemia and peripheral blood eosinophilia.

Case #1



Bone Marrow Biopsy





CASE 1 DIAGNOSIS

- Peripheral T-cell lymphoma (Angioimmunoblastic T-cell lymphoma) involving bone marrow and lymph node

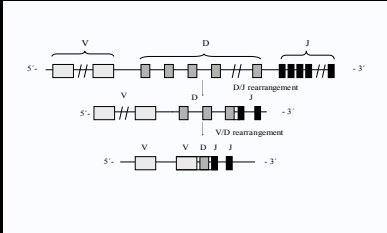
UTILITY OF MOLECULAR DIAGNOSTIC TESTING IN LYMPHOID PROLIFERATIONS

- To demonstrate evidence of clonality (reactive vs. neoplastic)
- To identify genetic abnormalities associated with a disease (classification)
- To identify viral associations with a disease

CLONALITY

- B and T lymphocytes normally undergo rearrangements of the immunoglobulin heavy chain gene or T cell receptor genes, respectively
- Detection of clonal rearrangements tends to correlate with malignancy
- Virtually all B and T cell neoplasms demonstrate gene rearrangements

OVERVIEW OF LYMPHOID RECEPTOR GENE REARRANGEMENTS

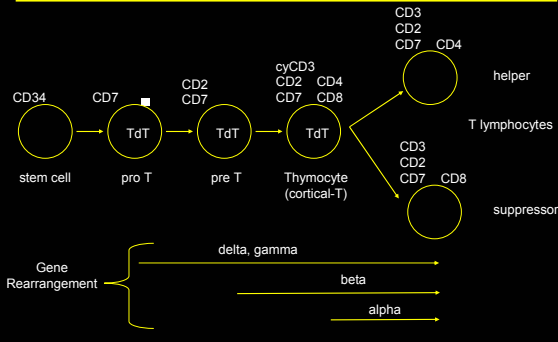


T-CELL RECEPTOR GENE REARRANGEMENTS

The T-cell receptor genes undergo rearrangements in the following sequence:

- TCR δ (14q11)
- TCR γ (7p15)
- TCR β (7q34)
- TCR α (14q11)

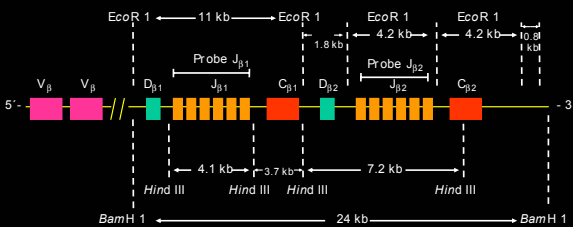
T-CELL DEVELOPMENT



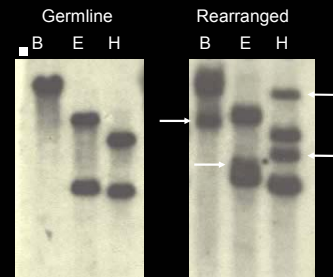
MOLECULAR GENETIC ABERRATIONS IN T-CELL PROLIFERATIONS

- T cell receptor β chain rearrangements
- T cell receptor γ chain rearrangements
- inv(14)(q12q32)
- Trisomy 8
- t(2;5) – *NPM/ALK*
- Epstein-Barr Virus
- HTLV-1

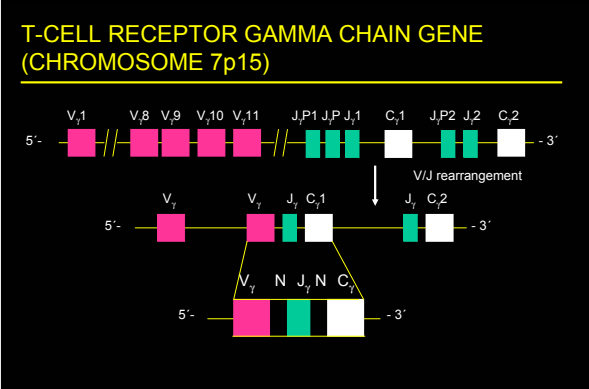
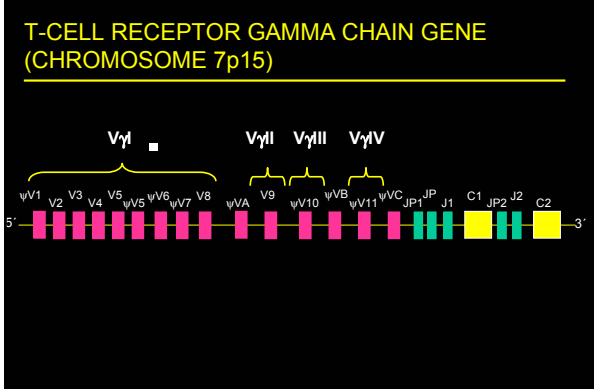
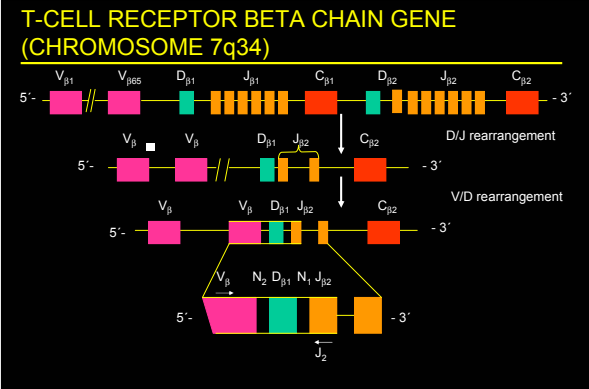
ENZYME RESTRICTION MAP OF THE GERMLINE T-CELL RECEPTOR BETA CHAIN GENE (7q34)



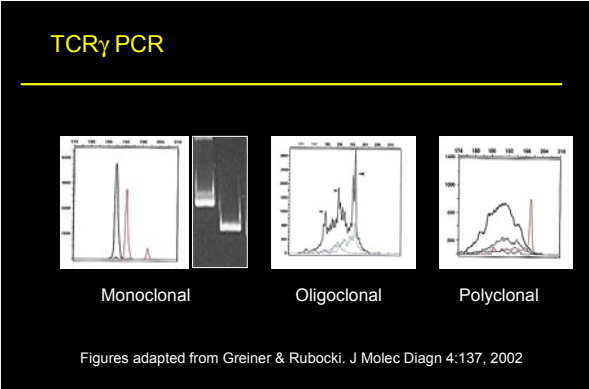
T CELL RECEPTOR β CHAIN GENE REARRANGEMENT ($J\beta 1/II$) SOUTHERN BLOT



Blot# 1213/1214



- ### ISSUES RELATED TO TCR γ PCR TESTING
- Gel-based analysis
 - Agarose
 - Polyacrylamide
 - DGGE, TGGE
 - Heteroduplex
 - GC clamp methods
 - Capillary electrophoresis
 - Number of primers
 - False Positive/False Negative results



SENSITIVITY OF DIFFERENT TCR γ PCR ASSAYS

Multi- institution Sample Exchange Data:

Primers	Frozen TP	Paraffin TP	Total TP
V γ 4 8J	74.3%	40.9%	61.4%
V γ 4 89/J	85.7%	100%	90.9%
V γ 4 810,11/J	92.9%	82.4%	88.9%
V γ 4 11/J	91.9%	71.1%	84.0%

Arber et al. J Molec Diagn 3:133, 2001

T-CELL RECEPTOR GAMMA CHAIN GENE (CHROMOSOME 7p15)

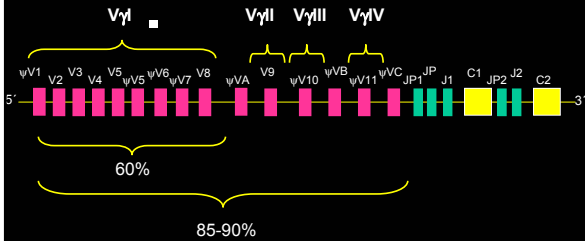
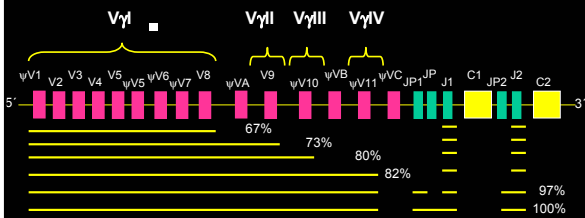


Table 4. Case Positivity Results for Different Primer Sets, Based on 60 Cases Selected with Previously Identified TCRγGR

Primer set	Positive cases
Vγ1-8, Jγ1/2	40 (67%)
Vγ1-8, Vγ9, Jγ1/2	44 (73%)
Vγ1-8, Vγ9, Vγ10, Jγ1/2	48 (80%)
Vγ1-8, Vγ9, Vγ10, Vγ11, Jγ1/2	49 (82%)
Vγ1-8, Vγ9, Vγ10, Vγ11, Jγ1/2, JPγ1/2	58 (97%)
Vγ1-8, Vγ9, Vγ10, Vγ11, Jγ1/2, JPγ1/2, JγP	60 (100%)

Lawnicki et al. J Molec Diagn 5:82, 2003

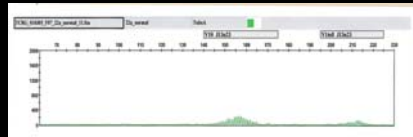
T-CELL RECEPTOR GAMMA CHAIN GENE (CHROMOSOME 7p15)



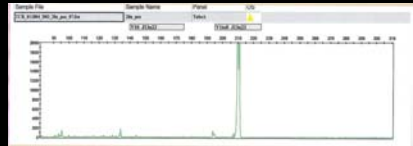
Lawnicki et al. J Molec Diagn 5:82, 2003

TCR GAMMA BIOMED-2 PCR

Reactive



Clonal



TCR GAMMA BIOMED-2 PCR

Reactive

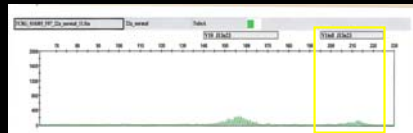


Clonal



TCR GAMMA BIOMED-2 PCR

Reactive



Clonal



T-CELL RECEPTOR FALSE NEGATIVE RESULTS

- Limited primer assays for PCR
- Inadequate DNA extracted (especially true from paraffin tissue samples)
- Sampling
- True natural killer (NK) cell origin

T-CELL RECEPTOR FALSE POSITIVE RESULTS

- Contamination
- Oligoclonal proliferations
- Small numbers of T cells (such as paraffin sections of small skin biopsies)
 - should give varying results on repeat assays
- Canonical rearrangements
 - Reported to occur with V γ 9/J γ P primers in γ/δ T cells

T-CELL RECEPTOR GENE REARRANGEMENTS IN OTHER DISEASES

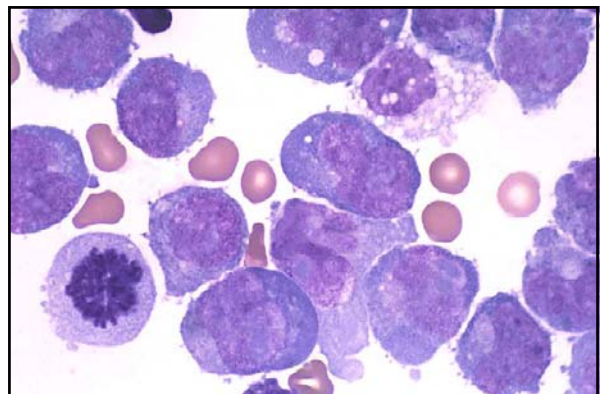
- “Preneoplastic” conditions
 - Angioimmunoblastic lymphadenopathy with dysproteinemia (AILD)
 - Lymphomatoid papulosis
- Lineage infidelity
 - Most common in precursor B cell lymphoblastic leukemia/lymphoma
 - May occur in other B cell lymphomas and rarely in non-lymphoid leukemias

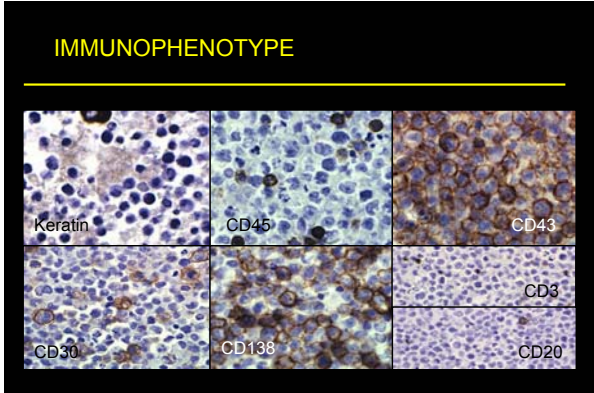
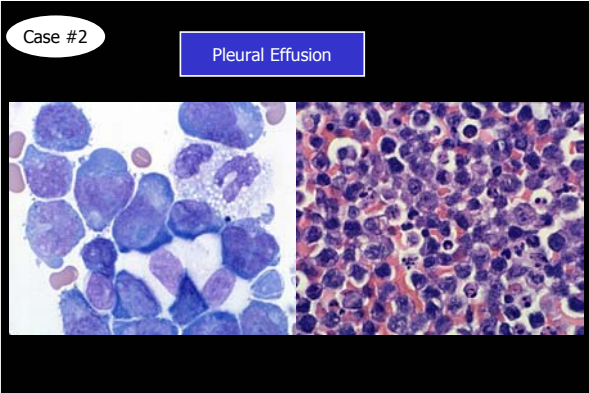
CASE 1 SUMMARY

- Differential diagnosis
 - Atypical hyperplasia versus lymphoma
- Immunohistochemistry
 - Atypical T-cell population identified
- Molecular studies
 - TCR γ gene rearrangement detected by PCR
- Final diagnosis
 - T-cell lymphoma

CASE 2

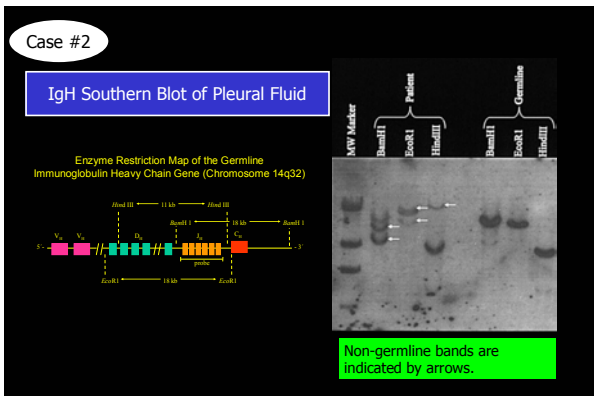
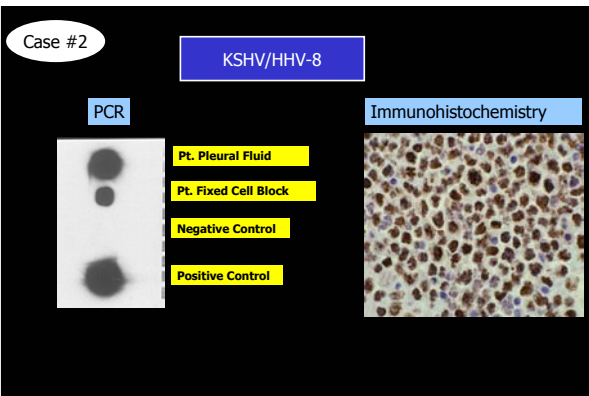
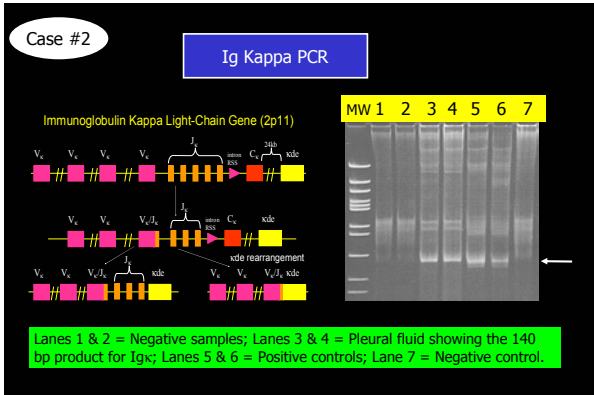
36-year-old man who underwent therapeutic thoracentesis of a left pleural effusion to alleviate acute dyspnea. He had no prior history of malignancy. However, he had a history of multicentric Castleman’s disease and HIV infection.





INITIAL MOLECULAR PROFILE

- IgH PCR Negative
- TCR γ PCR Negative



CASE 2 DIAGNOSIS

- Primary effusion lymphoma

PRIMARY EFFUSION LYMPHOMA (PEL)

- Most commonly occurs in young HIV-positive males with AIDS
- Serous body cavity effusion (pleural, pericardial or peritoneal cavities)
- No tumor mass, but individual pleomorphic tumor cells may be present on random pleural biopsies
- Pleomorphic/ immunoblast morphology

PRIMARY EFFUSION LYMPHOMA (PEL)

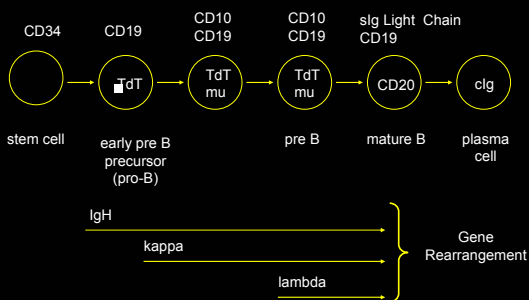
- Usually CD45 positive, but without expression of specific B or T lineage markers
- Monoclonal B lineage by molecular assays
- Usually EBV associated
- Always Kaposi-sarcoma herpes virus (KSHV)/HHV-8 associated

B-CELL RECEPTOR GENE REARRANGEMENTS

The B-cell receptor genes undergo rearrangements in the following sequence:

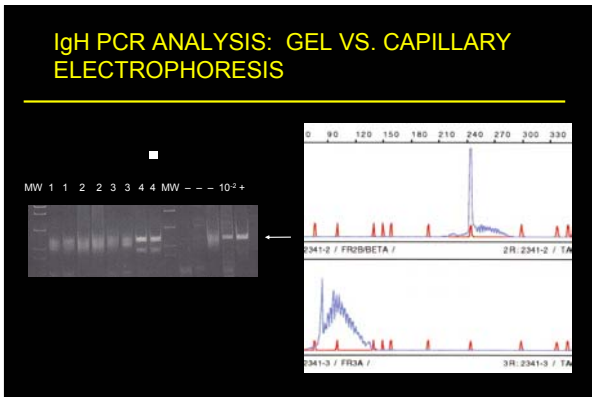
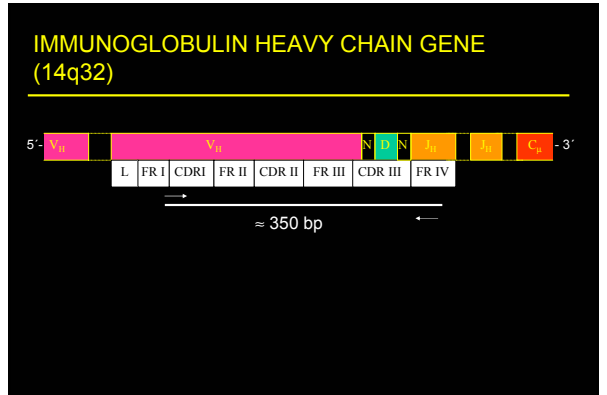
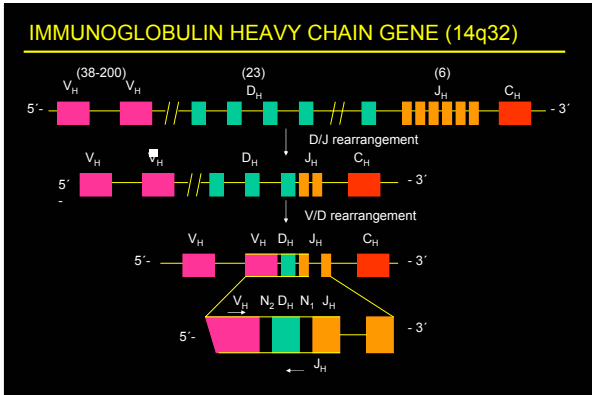
- IgH (14q32)
- Ig κ (2p11)
- Ig λ (22q11)

B-CELL DEVELOPMENT



MOLECULAR GENETIC ABERRATIONS IN B-CELL PROLIFERATIONS

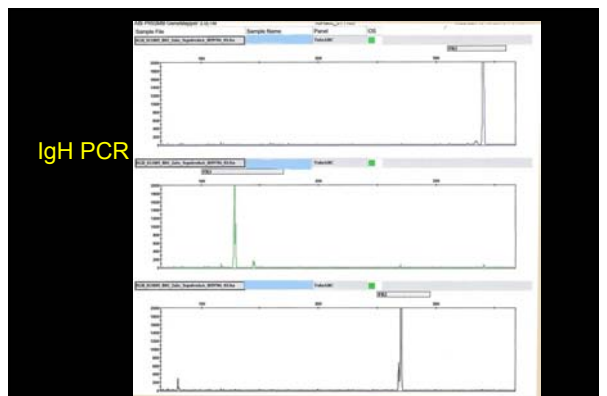
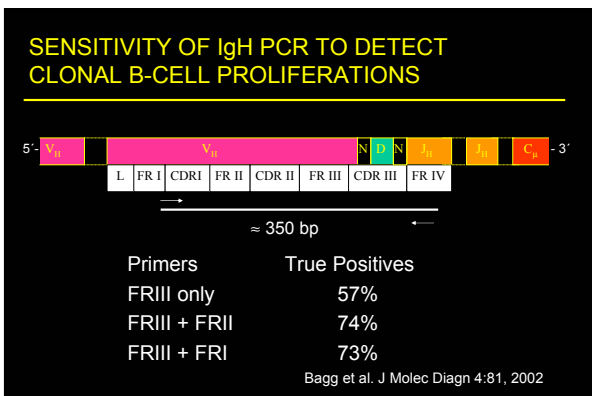
- Immunoglobulin heavy chain rearrangements
- Immunoglobulin light chain rearrangements
- t(14;18)- *JH/BCL2*
- t(11;14)- *JH/BCL1*
- t(11;18)- *API2/MALT1*
- c-MYC* and *BCL6* translocations
- KSHV/HHV8



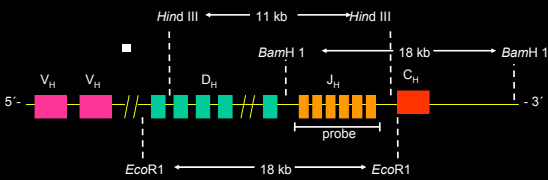
SENSITIVITY OF IgH PCR TO DETECT CLONAL B-CELL PROLIFERATIONS

- Follicular Lymphoma/ Plasma cell disorders: 35-55%
- Non-Follicular B-cell Lymphomas: 57-82%
- All B-cell Neoplasms: 60-70%

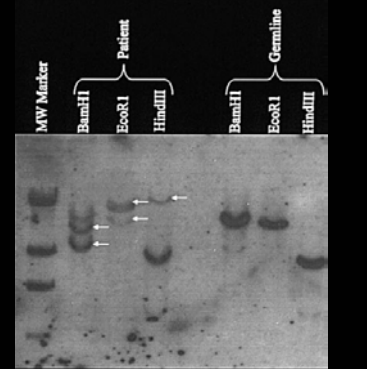
Abdel-Reheem et al. Arch Path Lab Med 120:357, 1996
Bagg et al. J Molec Diagn 4:81, 2002



ENZYME RESTRICTION MAP OF THE GERMLINE IMMUNOGLOBULIN HEAVY CHAIN GENE (14q32)



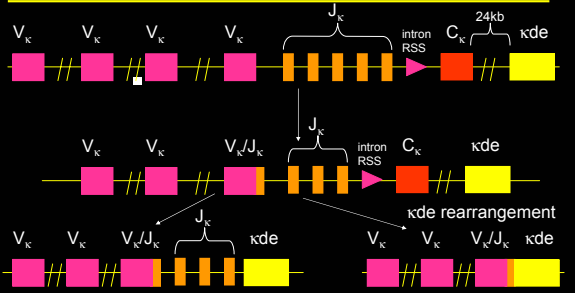
CASE 2 JH SOUTHERN BLOT



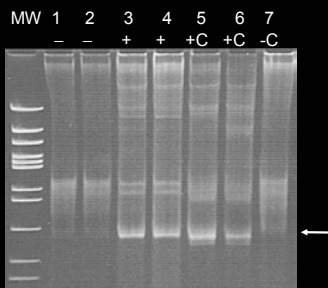
IMMUNOGLOBULIN KAPPA LIGHT CHAIN PCR (Igκ)

- Igκ gene rearrangements occur after IgH rearrangements and are present in virtually all mature B cell neoplasms
- Primers directed against the FR3 region of all six V_κ regions will detect clonality in 30-50% of mature B-cell neoplasms
- Useful as a second line test for IgH PCR negative proliferations (follicular lymphomas and plasma cell tumors)

IMMUNOGLOBULIN KAPPA LIGHT-CHAIN GENE (2p11)



IMMUNOGLOBULIN KAPPA LIGHT CHAIN PCR (Igκ)



IMMUNOGLOBULIN RECEPTOR FALSE NEGATIVE RESULTS

- Expected limitation of primers due to somatic hypermutation of IgH gene
- Inadequate DNA extracted (especially true from paraffin tissue samples)
 - The use of paraffin tissue resulted in an over 30% reduction in the detection of B cell clonality in one study
- Sampling

DEALING WITH IMMUNOGLOBULIN HEAVY CHAIN NEGATIVE RESULTS

- Use more than one IgH primer set
 - FRIII + FRII or FRIII + FRI
- Add JH/BCL2 testing if follicular lymphoma is suspected
- Consider adding Igκ testing, if available
- Go to Southern blot analysis if sufficient tissue is available
- Never assume that a negative IgH PCR test rules out a monoclonal B-cell proliferation

IMMUNOGLOBULIN RECEPTOR FALSE POSITIVE RESULTS

- Contamination
- Oligoclonal proliferations
- Small numbers of B cells (such as paraffin sections of small skin or GI biopsies)

CASE 2 SUMMARY

- Differential diagnosis
 - Undifferentiated malignant neoplasm
- Immunohistochemistry
 - Non-specific, but suggestive of hematolymphoid lineage
 - Evidence of KSHV/HHV8 infection
- Molecular studies
 - False negative IgH PCR result
 - B cell clonality confirmed by Igκ PCR and Southern blot
- Final diagnosis
 - B-cell lymphoma

Case #3

34 year old male patient

- Tiredness
- Splenomegaly
- CBC:

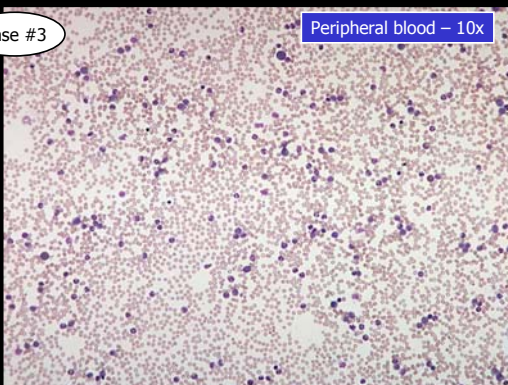
WBC: 125.6 x 10⁹/l
HCT: 33%
PLT: 231 x 10⁹/l

Normal cytogenetics

- what is the diagnosis and differential diagnosis?
- what is required to make the diagnosis?
- do we need molecular testing if cytogenetics is positive?
- do we need cytogenetics if molecular is positive?
- what's with all the molecular forms of the translocation?
- what is the role of testing post Rx?

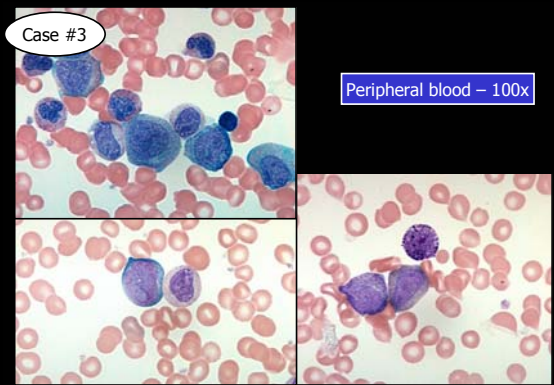
Case #3

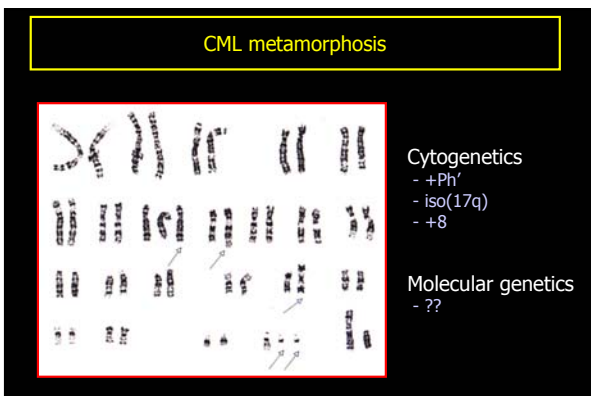
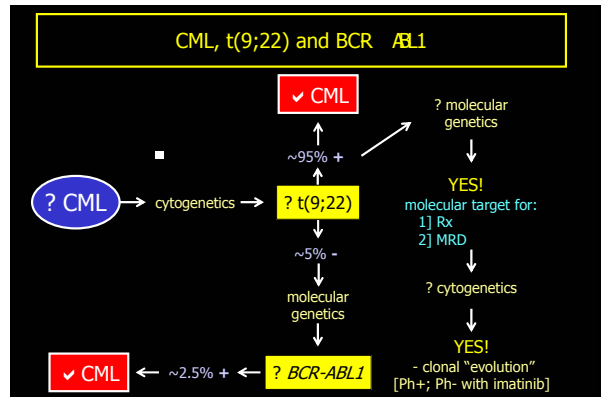
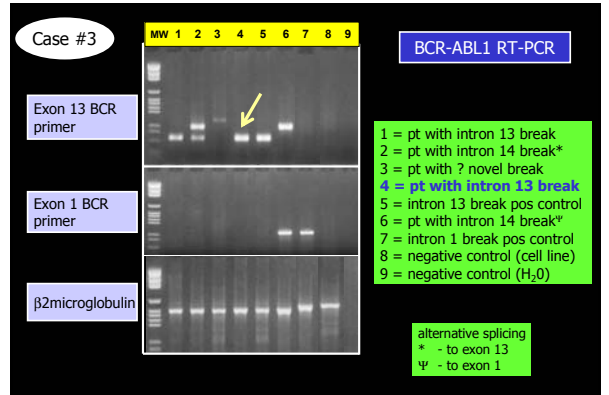
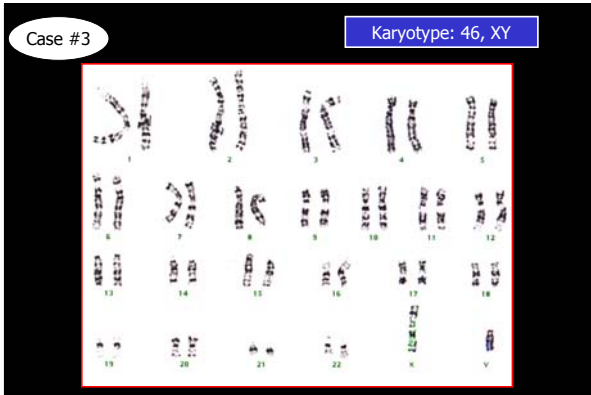
Peripheral blood – 10x



Case #3

Peripheral blood – 100x





- When to order BCR ABL1 RT PCR
- DIAGNOSIS**
- CML vs leukemoid reaction
 - CML vs other MPDs
- PROGNOSIS**
- ALL - see later ...
- MINIMAL RESIDUAL DISEASE**
- CML
 - ALL
- CAVEATS**
- presence in "normals" [but at very low levels]
 - ET [but ought to distinguish]

What's going on at the DNA level ...

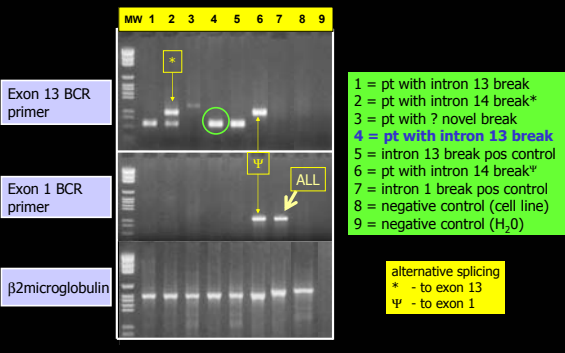


... and what do we do at the RNA/cDNA level ...



Minimal residual disease testing

- Contexts:
 - stem cell transplantation: for early relapse
 - imatinib mesylate (and IFN α): to gauge response
- Peripheral blood as good as bone marrow
- Single qualitative result typically not predictive
- Positivity in 1st 6 months post SCT: **not predictive**
- Positivity after 1st 6 months post SCT: **predictive**



Minimal residual disease testing

- Quantitative RT PCR (RQ-PCR) likely to change everything
 - declining levels \rightarrow predict remission
 - rising levels \rightarrow predict relapse
- What defines a meaningful change?
 - 1-log [10-fold] change over 3 or more consecutive time-points
 - >3-log [1,000-fold] \downarrow over 12 months on imatinib: stable disease
- Is there a magic number?
 - ? 0.02% \rightarrow 1:10,000
- Frequency of testing: \sim once every 3 months \rightarrow annually
- Does MRD detection help the patient? ...probably (DLI Rx)

Molecular and other testing in non CML MPDs

	karyotype	genes	PRV1	EEC	\downarrow mpl megas	\downarrow GATA1 megas	\uparrow circ CD34+
PV	9p+, +8+9	?	+	+	+	?	-
ET	?	?	[50%]	[50%]	+/-	?	-
IMF	del(13q14)	?	-	-	+/-	+	+
PPMF	1q+	?	?	?	?	?	?
CEL	cryptic del(4q12)	fip111-pdgfra	?	?	?	?	?
SM	cryptic	c-kit 816	?	?	?	?	?
CNL	+/- t(9;22)	μ bcr-abl	?	?	?	?	?

Molecular and other testing in non CML MPDs

	karyotype	genes	PRV1	EEC	↓ mpl megas	↓GATA1 megas	↑ circ CD34+
PV	JAK2 V617F mutation						
ET							
IMF							
PPMF							
CEL	cryptic del(4q12)	fip111- pdgfra	?	?	?	?	?
SM	cryptic	c-kit 816	?	?	?	?	?
CNL	+/- t(9;22)	μbcr- abl	?	?	?	?	?

Case #3

Summary

- * what is the diagnosis and differential diagnosis?
 - CML versus leukemoid reaction (cytogenetics were normal)
- * what is required to make the diagnosis?
 - RT-PCR for BCR ABL1 (it defines the disease)
- * do we need molecular testing if cytogenetics is positive?
 - yes! (target to treat and track)

Case #3

Summary

- * do we need cytogenetics if molecular is positive?
 - yes! (for Ph+ metamorphosis, and Ph- clones with imatinib Rx)
- * what's with all the molecular forms of the translocation?
 - alternative M₁ breakpoint and splicing (? significance)
- * what is the role of testing post Rx?
 - significant

Case #4

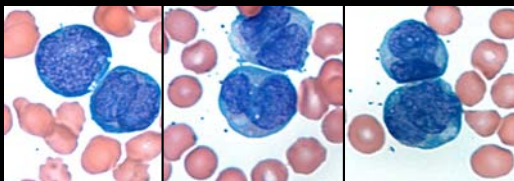
28 year old female patient

- Fever and malaise
- Numerous petechiae
- CBC:
 - WBC: "elevated"
- Smear:
 - numerous abnormal cells

- what is the diagnosis and differential diagnosis?
- what more is required to make the diagnosis?
- is a specific cytogenetic abnormality suspected?
- what is your preference: cytogenetics or molecular genetics?
- are there other cyto-/molecular genetic abnormalities to consider?
- what is the significance of these "variant" translocations?

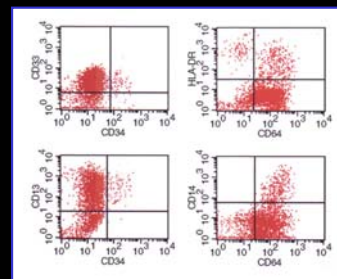
Case #4

Peripheral blood – 100x



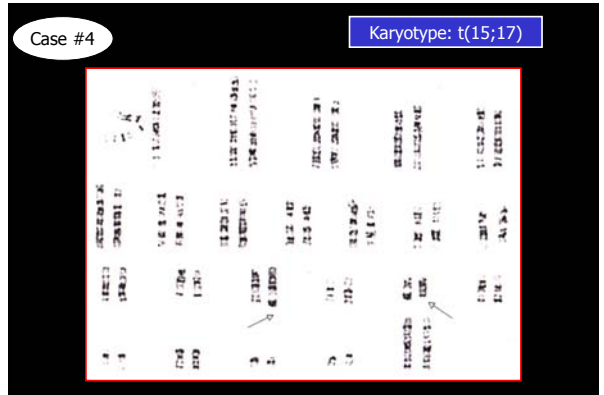
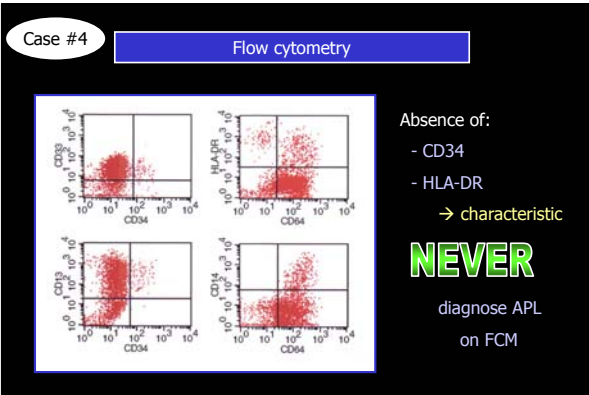
Case #4

Flow cytometry



Absence of:

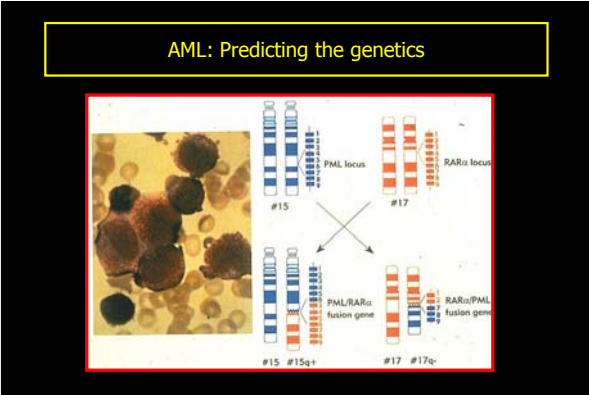
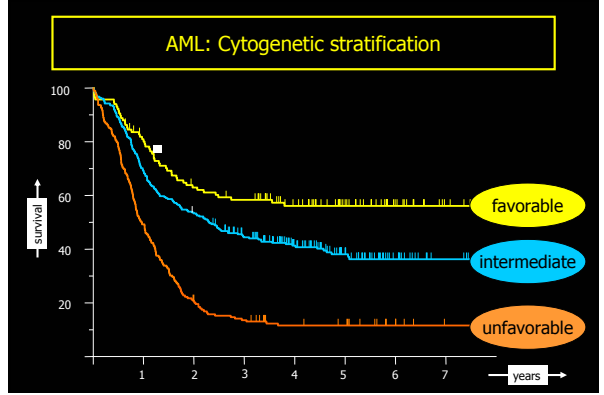
- CD34
 - HLA-DR
- characteristic



AML: Cytogenetic stratification

Risk group	Genetics	Frequency	CR	5yr Survival
Favorable	t(8;21) t(15;17) inv(16)	~25% (~5%)	~85% (~70%)	~60% (~35%)
Intermediate	normal +8, +21 11q23	~50% (~63%)	~80% (~60%)	~40% (~13%)
Unfavorable	-5, -7 3q complex	~25% (~32%)	~60% (~25%)	~15% (~2%)

Combined data from CALGB, UK-MRC and US-intergroup for "young" AML (% for >55 or 60)

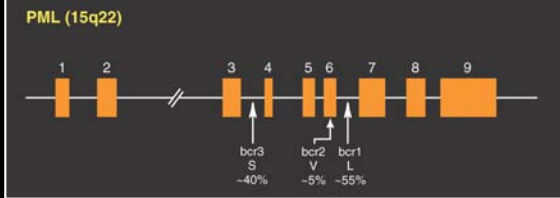


The various flavors of APL

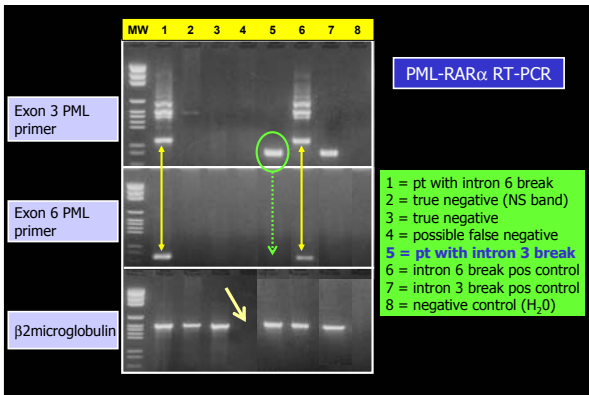
Translocation	Partner	Frequency	ATRA-responsive
t(15q22;17q21)	PML	>99%	+
t(11q23;17q21)	PZLF	0.5%	-*
t(11q11;17q21)	NuMA	<0.5%	?+
t(5q32;17q21)	NPM	<0.5%	?+
t(17q21;17q21)	STAT5b	<0.5%	?

RARα mutations → ATRA resistance
 [* Rx: HDAC]

What's going on at the DNA level ...



... and what do we do at the RNA/cDNA level ...



AML: Cytogenetics

The big 4 \rightarrow *sine qua non* for WHO

- t(15;17) [M3] ~10- 15%
 - t(8;21) [M2] ~10- 15%
 - inv(16) [M4Eo] ~10- 15%
- GOOD
- t(11q23)* [M4/M5] ~5- 10%
- not so good[†]

* promiscuous (>30 partners); FAB for translocations only - PTD [M1/M2]
† ? exception = t(9;11)

AML: Cryptic abnormalities

inv(16)

- ~ 30% of positive cases lack M4Eo morphology
- upto 30% with M4Eo morphology are:
 - + RT-PCR
 - cytogenetics

t(8;21)

- upto 30% are:
 - + RT-PCR
 - cytogenetics

t(15;17)

- upto 15% are:
 - + RT-PCR
 - cytogenetics

need for alternative gene products to use in AML [big 4 < 50%]

AML: Cryptic abnormalities

MLL PTD

WT1

FLT3

CEBPA

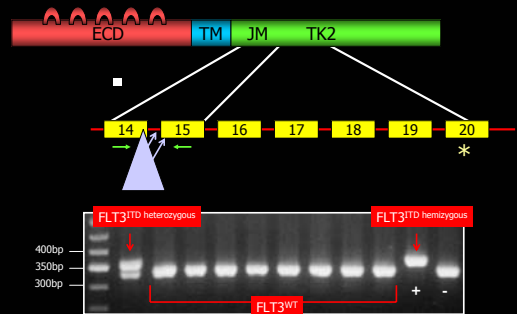
BAALC

NPM

FLT3

- 2 types of genetic abnormalities described:
 - ITD of JM region ~23% PCR
 - Asp835 missense mutation ~7% RE-PCR
 - ~30%
- thus one of the commonest genetic targets in ANLL
 - constitutive ON switch

FLT3 genotyping



FLT3

- Commonest identified molecular target in ANLL
- Possibly most prognostically relevant molecular lesion
 - quantification might be important (mutant:wt)
 - ? add to cytogenetic stratification groups (? also CEBPA - good - ~10%)
- A potentially useful MRD target
 - patient specific primers vs ITD
 - ITD may not be stable between presentation and relapse [+ → -] [- → +] [+^h → +^h] [+ → ++]
- A potential therapeutic target
 - a la imatinib mesylate in CML

AML: Cytogenetics vs Molecular

- each has advantages and disadvantages
 - molecular fewer false negatives [diagnostic sensitivity]
 - cytogenetics more global [see not just what is being PCR'd]
 - some lesions never seen cytogenetically [FLT3]
- complement rather than supplement one another
- even with + cytogenetics, need molecular for:
 - molecularly targeted Rx [ATRA]
 - subsequent MRD [leukemia fingerprint]

AML: FISH vs Molecular

- each has advantages and disadvantages
 - diagnostic sensitivity: FISH ≈ molecular (probes bigger than primers)
 - analytic sensitivity: molecular > FISH (MRD testing)
 - numeric abnormalities: FISH > molecular (? array CGH)

Case #4

Summary

- * what is the diagnosis and differential diagnosis?
 - microgranular APL vs monoblastic leukemia [don't miss the former]
- * what more is required to make the diagnosis?
 - genetics [molecular genetics > cytogenetics]
- * is a specific cytogenetic abnormality suspected?
 - t(15;17) [seen in ~99% of cases]

Case #4 **Summary**

- * what is your preference: cytogenetics or molecular genetics?
 - both please [~15% missed by cytogenetics]
- * are there other cyto-/molecular genetic abnormalities to consider?
 - of course! [variant translocations]
- * what is the significance of these "variant" translocations?
 - some not ATRA sensitive [and may need other targeted Rx]

Case #5 **8 year old female patient**

- Severe pharyngitis
- Generalized lymphadenopathy
- CBC:
 - WBC: 27.3 x 10⁹/l
 - Hb: 8.3 g/dl
 - Plt: 39 x 10⁹/l

- what immunophenotype might be predicted?
- what are some of the the immunophenotype-genetic correlates?
- what is the relevance of the various cytogenetic abnormalities?
- if cytogenetics were normal, how would you proceed?
- what is the rationale for performing molecular assays in this case?

Case #5 **Peripheral blood – 100x**

Case #5 **Flow cytometry**

Expect:

- 1) lymphoblastic
- 2) B-lineage
- 3) precursor B-cell

Case #5 **Karyotype: 46, XX**

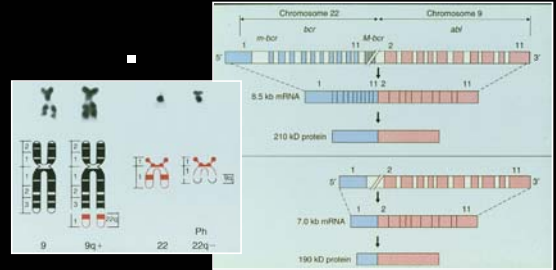
Case #5 **FISH**

BCR breakpoints



- ~100% CMLs involve M-bcr
- ~60% adult ALLs involve m-bcr, but ~40% involve M-bcr

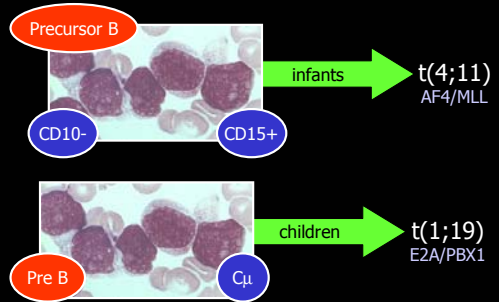
t(9;22) CML versus ALL



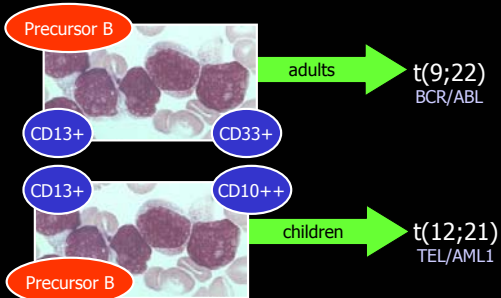
t(12;21)

- commonest translocation in pediatric ALL
 - ~25% of cases
 - 2-5 years of age
- favorable prognosis
 - in most, but not all, studies (? late relapses)
- typically associated with:
 - relatively low WBC count (<50)
 - normal ploidy (DNA =1)
- true cryptic abnormality
 - must do FISH or RT-PCR to detect

ALL: Predicting the genetics



ALL: Predicting the genetics



ALL: Cryptic genetic abnormalities

- TEL(ETV6)/AML1(CBFα2) chimera
 - ~ "never" detected cytogenetically
 - better prognosis (? less Rx)
 - goodness controversial
 - ? similar incidence in relapses ?
- Precursor B ALL
- t(12;21)
 - Pediatrics (~20%)
 - t(9;22)
 - Adults (~25%)
 - BCR/ABL1 chimera
 - ~ 10% missed cytogenetically
 - worse prognosis (more Rx: early SCT)

Other molecular abnormalities in ALL

CDKIs

- inhibit progression through G₁
- act as tumor suppressor genes
- two major families (INK4, CIP/KIP)
- INK4 ↓ in >50% T-ALL
- mechanisms:
 - point mutation
 - deletion
 - transcriptional silencing
- ? relevance
 - generally poor prognosticators (p16, p21)

del1p32

- submicroscopic fusion TAL1/SCL1/TCL5 and SIL
- commonest fusion in T-ALL ~25%

notch1

- commonest mutation in T-ALL ~50%

Case #5

Summary

- * what immunophenotype might be predicted?
 - precursor B cell
- * what are some of the immunophenotype-genetic correlates?
 - "characteristic" in t(12;21), t(9;22), t(1;19), t(4;11)
- * what is the relevance of the various cytogenetic abnormalities?
 - generally poor [with the exception of t(12;21)]

Case #5

Summary

* if cytogenetics were normal, how would you proceed?

- RT-PCR [multiplex, or targeted – dictated by age]

* what is the rationale for performing molecular assays in this case?

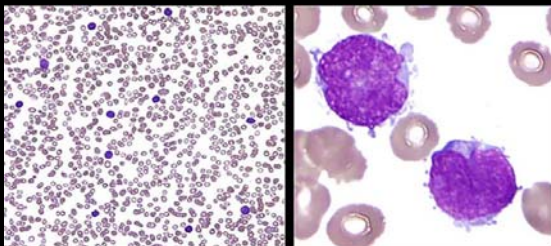
- prognosis, therapy, fingerprint for MRD

CASE 6

67 year old male patient referred to hematology clinic with a reported diagnosis of "early" chronic lymphocytic leukemia. Physical examination revealed generalized lymphadenopathy, and a CBC showed a WBC of $9.7 \times 10^9/l$, a hemoglobin of 10.1g/dl and platelet count of $87 \times 10^9/l$.

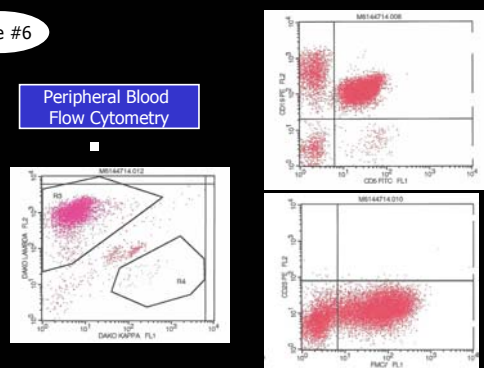
Case #6

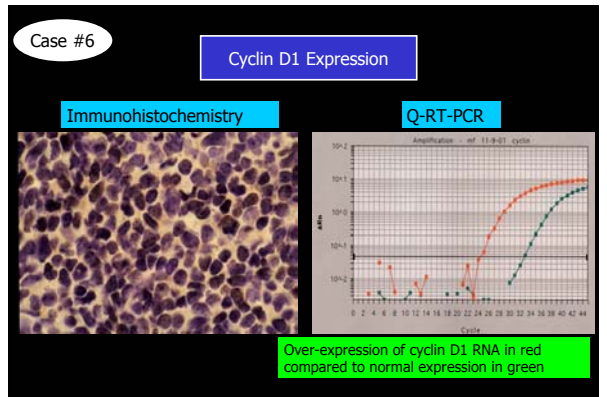
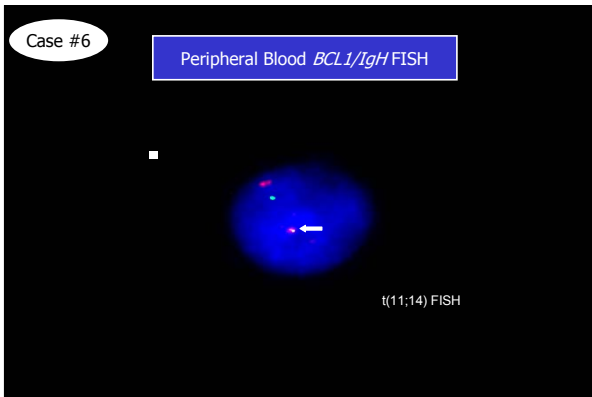
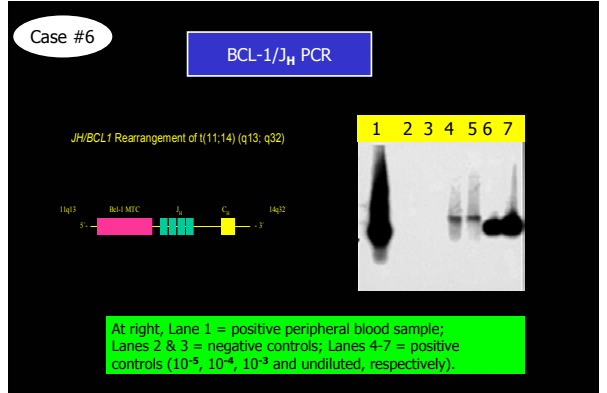
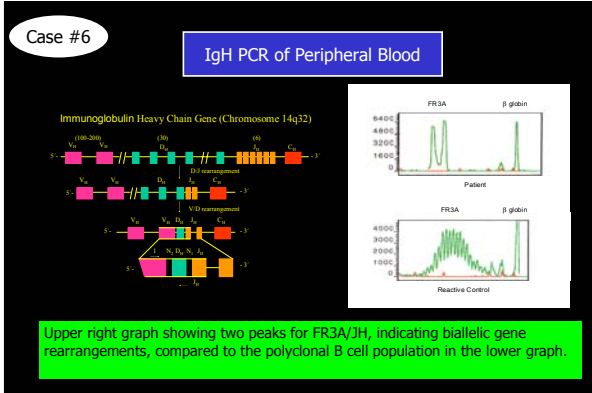
Peripheral Blood



Case #6

Peripheral Blood Flow Cytometry





CASE 6 DIAGNOSIS

Peripheral blood involvement by mantle cell lymphoma

UTILITY OF MOLECULAR DIAGNOSTIC TESTING IN LYMPHOID PROLIFERATIONS

- To demonstrate evidence of clonality (reactive vs. neoplastic)
- To identify genetic abnormalities associated with a disease (classification)
- To identify viral associations with a disease

MOLECULAR GENETIC ABERRATIONS IN SMALL B-CELL PROLIFERATIONS

Follicular lymphoma	t(14;18) - <i>JH/BCL2</i>
Mantle cell lymphoma	t(11;14) - <i>JH/BCL1</i>
Marginal zone lymphoma	t(11;18) - <i>API2/MALT1</i>

MANTLE CELL LYMPHOMA

- A proliferation of monoclonal, CD5-positive small B lymphocytes that lack CD23 expression
- Virtually all cases demonstrate immunoglobulin heavy chain gene rearrangements
- The t(11;14)(q13;q32) involving the *CYCLIN D1* gene on chromosome 11 is present in the majority of cases

MANTLE CELL LYMPHOMA

- 40-50% of cases translocations involving the major translocation cluster (MTC) region of t(11;14)
- Over 90% of cases are reported to over express cyclinD1, either at the protein or mRNA level
- Virtually all cases can be detected by FISH analysis

JH/BCL1 REARRANGEMENT OF t(11;14) (q13; q32)



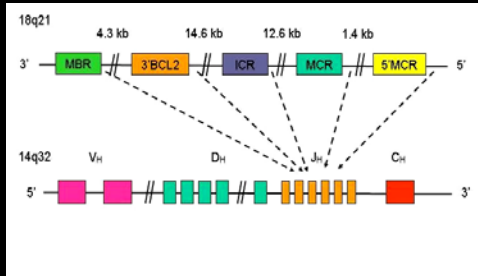
FOLLICULAR LYMPHOMA

- A proliferation of monoclonal B cells that frequently express CD10 and lack CD5
- Virtually all cases demonstrate immunoglobulin heavy chain gene rearrangements by Southern blot analysis
- Depending on the method used, 45-65% of cases will not demonstrate an immunoglobulin heavy chain rearrangement by PCR analysis due to somatic hypermutation of the immunoglobulin heavy chain gene

FOLLICULAR LYMPHOMA

- Approximately 80% of cases have evidence of t(14;18)(q32;q21) involving the *BCL2* gene on chromosome 18
- Most translocations involve the major breakpoint region (MBR) of t(14;18)
- The remaining translocations involve minor cluster regions (*MCR*, *3'BCL2*, *5'mcr*) of t(14;18)
- *BCL6* translocations (up to 24%) associated with large cell morphology/transformation

FOLLICULAR LYMPHOMA *BCL2* BREAKPOINTS

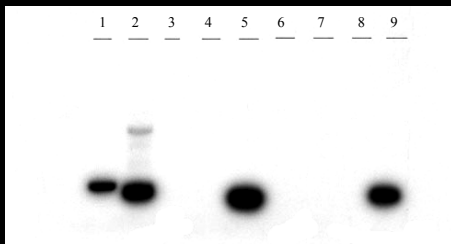


FREQUENCY OF *BCL2* BREAKPOINTS IN FOLLICULAR LYMPHOMA (n=236)

<i>MBR</i>	50%
<i>ICR</i>	13.6%
<i>3'BCL2</i>	5.5%
<i>MCR</i>	4.7%
<i>5'MCR</i>	1.3%
Two Breakpoints	3.4%
Total <i>BCL2</i> Positive	71.6%

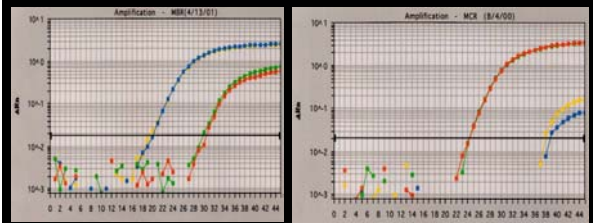
Shum & Arber J Molec Diagn 6:418, 2004

JH/BCL2 QUALITATIVE PCR



JH/BCL2 PCR for the major breakpoint region (MBR), showing a positive result in lanes 1, 2, 5 and 9.

JH/BCL2 QUANTITATIVE PCR



JH/BCL2 FISH



Nucleus with normal pattern of 14q32 and 18q21 Representative nucleus displaying $t(14;18)(q32;q21)$

MARGINAL ZONE LYMPHOMA

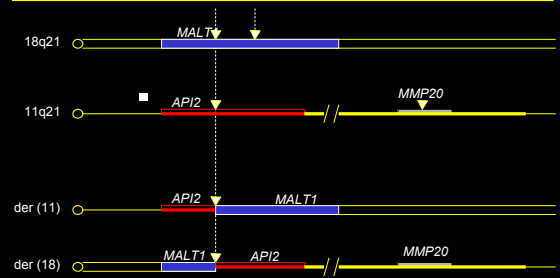
- A proliferation of monoclonal B cells that lack CD5 and CD10 expression
- $t(11;18)(q21;q21)$ is the most common balanced translocation in marginal zone lymphoma
- This translocation involves the apoptosis inhibitor gene (*API2*) on chromosome 11 and the *MALT1* gene on chromosome 18
- Other translocations involving *MALT1*, *IgH*, *BCL10* and *BCL6* may also occur
- Trisomies, especially of chromosome 3 and 18, often occur in the translocation negative cases

MOLECULAR GENETICS OF EXTRANODAL MARGINAL ZONE LYMPHOMA

IgH/ Igκ	100%	
t(11;18), <i>API2/MALT1</i>	13.5%	Lung, GI
t(14;18), <i>IGH/MALT1</i>	10.8%	Ocular, skin, s.g
t(1;14), <i>BCL10/IGH</i>	1.6%	Various
t(14;18), <i>JH/BCL2</i>	0%	
t(11;14), <i>JH/BCL1</i>	0%	
Trisomy 3	30.9%	Various
Trisomy 18	11.2%	Various

Data adapted from Streubel et al. Leukemia 18:1722, 2004

t(11;18)(q21;q21) IN MARGINAL ZONE LYMPHOMA



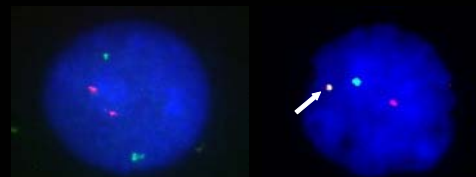
Adapted from Dierlamm et al. Blood 93:3601, 1999

t(11,18) RT-PCR IN MALT LYMPHOMA



Lanes 2 and 7 Show Evidence of t(11,18)

DETECTION OF t(11;18) BY FISH



Nucleus with normal pattern of 11q22 and 18q21

Representative nucleus displaying t(11;18)

Image provided by Marilyn Slovak, Ph.D.

TRISOMY 3 IN MALT LYMPHOMA



Negative nucleus

Trisomy 3

Image provided by Marilyn Slovak, Ph.D.

BCL-10

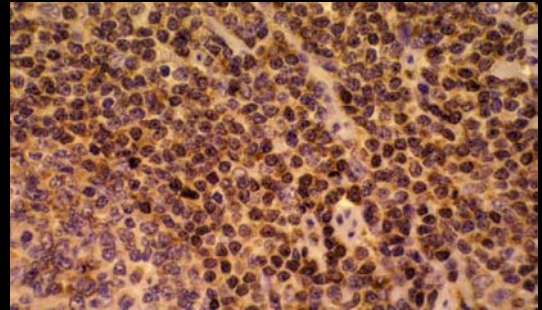
- An apoptotic regulatory molecule
- Expressed (cytoplasmic only) in normal lymphoid cells
- Encoded by a gene at 1p22
- t(1;14)(p22;q32) originally thought to occur in ~10% of MALT lymphomas, but frequency seems much lower in recent studies
- Truncated bcl-10 loses its pro-apoptotic activity, but retains ability of NF-κB activation for increased cell survival

BCL-10 IN LYMPHOID TISSUES

	Cytoplasmic	Nuclear
Normal lymphoid tissue n=31	100%	0%
MALT lymphoma n=40	100%	60%
Follicular lymphoma n=21	90%	10%
Mantle cell lymphoma n=17	100%	0%
Nodal DLBCL n=18	78%	22%
Mucosal DLBCL n=20	100%	0%

Ye et al. Am J Pathol 157:1147, 2000

BCL-10 IMMUNOHISTOCHEMISTRY



CORRELATION BETWEEN BCL-10 PROTEIN EXPRESSION AND t(11;18)

		t(11;18)	
		+	-
Nuclear BCL-10	+	20	7
	-	0	23

Liu et al. Blood 98:1182, 2001

CHRONIC LYMPHOCYTIC LEUKEMIA AND SMALL LYMPHOCYTIC LYMPHOMA

- A proliferation of monoclonal small B cells with CD5 and CD23 expression
- Associated with various cytogenetic aberrations (del(13q14), del(11q23), +12, del(17p13)) that have prognostic significance and are best detected by FISH analysis
- Can also be separated into pre- and post-germinal center types

FISH ANALYSIS OF CLL (n=325)

Probe	Frequency	Prognosis
13q del	55% (36% single)	Good
11q del	18%	Poor
Trisomy 12	16%	Intermediate
17p deletions	7%	Poor
Other abn	18%	
Total abn	82%	
Normal	18%	Intermediate

Döhner et al N Engl J Med 343:1910, 2000

CHRONIC LYMPHOCYTIC LEUKEMIA AND SMALL LYMPHOCYTIC LYMPHOMA

- | | |
|----------------------------------|-------------------------------|
| • Pre-Germinal Center | • Post-Germinal Center |
| – non-mutated IgH (naïve B cell) | – mutated IgH (memory B cell) |
| – CD38 positive | – CD38 negative |
| – ZAP70 positive | – ZAP70 negative |
| – poorer prognosis | – better prognosis |

LYMPHOPLASMACYTIC LYMPHOMA

- A proliferation of monoclonal B cells with plasmacytoid differentiation that usually lack CD5 and CD10 expression
- t(9;14)(p13;q32) was originally reported to be present in approximately 50% of lymphoplasmacytoid lymphomas

LYMPHOPLASMACYTIC LYMPHOMA

- The translocation involves the *PAX5* gene of chromosome 9 and the switch μ region of the Ig heavy chain locus on chromosome 14
- *PAX5* normally encodes a B cell specific transcription factor (BSAP) that is involved in the control of B cell proliferation and differentiation
- *PAX5* translocations, however, *appear to be uncommon in lymphoplasmacytic lymphomas*

CASE 6 SUMMARY

- Differential diagnosis
 - CLL vs. reactive vs. other lymphoma
- Flow cytometry
 - CD5-positive, monotypic B cell population
- Molecular studies
 - B cell clonality confirmed by IgH PCR
 - t(11;14) detected by PCR and FISH
 - cyclin D1 over expression detected by immunohistochemistry and Q-RT-PCR
- Final diagnosis
 - Peripheral blood involvement by mantle cell lymphoma

THE ROUTINE USE OF MOLECULAR PATHOLOGY ASSAYS

- Up-front diagnosis
- Classification
- Determination of prognosis
- Post therapy monitoring

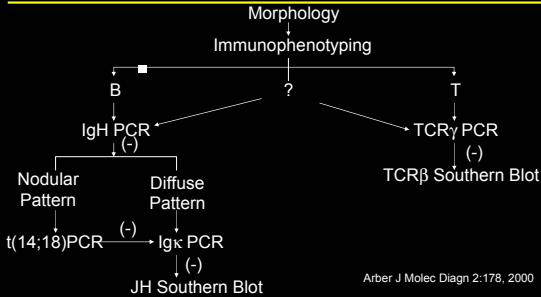
DIAGNOSIS

- Molecular assays should never be interpreted as stand-alone diagnostic tests
- Results must be correlated with all other available information, including clinical features, morphology, and immunophenotyping

LYMPHOMA DIAGNOSIS

- The majority of cases can be diagnosed without the use of molecular assays
- These assays can be useful in atypical proliferations, especially to exclude T cell lymphoma
- Antigen receptor gene rearrangement studies are usually sufficient to determine lymphoid cell clonality
- Addition of Ig κ or t(14;18) PCR may be useful due to the high false negative rate for IgH PCR in some specimen types
- A negative molecular study does not exclude malignancy

DIAGNOSTIC ALGORITHM FOR CLONALITY MOLECULAR TESTING IN LYMPHOID PROLIFERATIONS



LEUKEMIA DIAGNOSIS

- Chronic myelogenous leukemia
 - t(9;22) or *BCR/ABL*
- WHO classification of AML
 - AML with t(8;21) or *AML1/ETO*
 - AML with inv(16)/t(16;16) or *CBFβ/MYH11*
 - AML with t(15;17) or *PML/RARα* and variants
 - AML with 11q23 (*MLL*) abnormalities

LYMPHOMA CLASSIFICATION

- Lymphomas of small B-cells
 - Follicular lymphoma and t(14;18) or *JH/BCL2*
 - Mantle cell lymphoma and t(11;14) or *JH/BCL1*
 - MALT lymphoma and t(11;18) or *API2/MALT1*
- Other lymphomas
 - *ALK* translocations in anaplastic large cell lymphoma
 - *CMYC* translocations in Burkitt/Burkitt like lymphoma
 - EBV integration in nasal type NK/T cell lymphoma
 - KSHV in primary effusion lymphomas

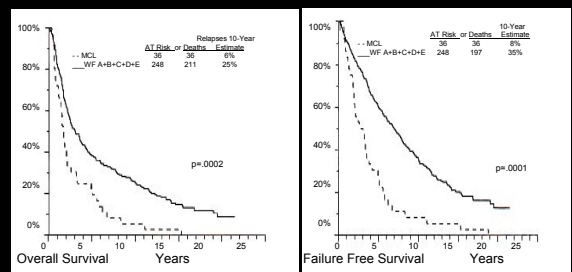
LEUKEMIA CLASSIFICATION

- Chronic myeloproliferative disorders
 - Presence of t(9;22) or *BCR/ABL* is definitional for CML
 - Absence of t(9;22) or *BCR/ABL* is required for diagnosis of other chronic myeloproliferative disorders or mixed MDS/MPD diseases
- Acute leukemias
 - Genetic categories of AML are now part of the WHO classification
 - Genetic categories of ALL may be included in future classifications

LYMPHOMA PROGNOSIS

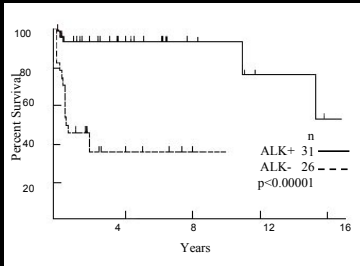
- Poor prognosis of mantle cell lymphoma versus other lymphomas of small B lymphocytes
- Resistance of *API2/MALT1* positive MALT lymphoma to *H. pylori* therapy
- Prognosis of FISH and IgH mutation status in CLL
- Improved survival after chemotherapy for germinal center B-cell like diffuse large B cell lymphoma
- Improved survival for *ALK*-positive anaplastic large cell lymphoma

MANTLE CELL LYMPHOMA SURVIVAL



Fisher et al. Blood 85:1075, 1995

OVERALL SURVIVAL OF T-CELL NULL ALCL BASED ON ALK PROTEIN EXPRESSION

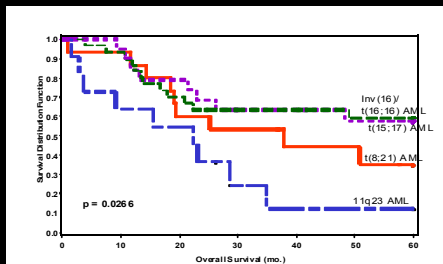


Gascoyne et al. Blood 93:3913, 1999

ACUTE MYELOID LEUKEMIA PROGNOSIS

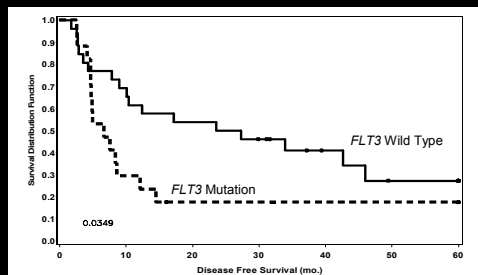
- Favorable
 - AML with t(8;21) or *AML1/ETO*
 - AML with inv(16)/t(16;16) or *CBF β /MYH11*
 - AML with t(15;17) or *PML/RAR α* and variants
- Unfavorable
 - AML with 11q23 (*MLL*) abnormalities
 - AML with *FLT3* mutations
 - AML with complex karyotypes

OVERALL SURVIVAL OF AMLs WITH RECURRENT CYTOGENETIC ABNORMALITIES



Arber et al. Am J Clin Pathol, 119:672, 2003.

IMPACT OF *FLT3* MUTATIONS ON DISEASE FREE SURVIVAL OF AML WITH NORMAL CYTOGENETICS



PRECURSOR B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA PROGNOSIS

- Favorable
 - ALL with t(12;21) or *TEL/AML1*
 - Hyperdiploidy
- Unfavorable
 - ALL with t(9;22) or *BCR/ABL*
 - ALL with t(4;11) or *AF4/MLL*
 - ALL with t(1;19) or *PBX/E2A*

MINIMAL RESIDUAL DISEASE TESTING (1)

- Antigen receptor gene rearrangements by PCR
 - Use consensus primers that limit the level or detection (1 in 100)
- Specific translocation assays by PCR or RT-PCR
 - Can detect very low levels of the translocation (1 in 100,000 to 1 in 1,000,000)
 - Some translocations, especially t(8;21) and inv(16), may persist after therapy with no prognostic significance
 - Serial quantitative PCR assays may be more useful for the evaluation of patients with these abnormalities

MINIMAL RESIDUAL DISEASE TESTING (2)

- Patient specific PCR
 - Directed against the tumor specific antigen receptor gene rearrangement
 - May detect very low levels residual disease
 - Will not detect new or second clones
 - Labor intensive
- FISH analysis
 - Most assays cannot reliably detect disease below 5%
 - Newer methods allow lower levels of disease detection and may be more useful for minimal residual disease detection

MOLECULAR PITFALLS - FALSE POSITIVES

- Contamination
 - Use of proper controls and techniques is essential
- Antigen receptor assays on small samples or samples with few lymphocytes
 - Pseudoclonality may occur due to selective amplification
 - Result should not be reproducible, especially using precise methods that size the gene rearrangement
- Antigen receptor assays in oligoclonal proliferations
 - Often associated with infections or autoimmune disease
 - Result should not be reproducible, if run in duplicate

MOLECULAR PITFALLS - FALSE POSITIVES

- Detection of translocations in “normal” patients
 - Very low levels of leukemia or lymphoma associated translocations can be detected in some individuals with no clinical significance
 - Usually found with qualitative PCR assays that use a very high number of amplifications
 - Testing can be optimized to avoid this level of detection
 - Serial quantitative assays may be useful to evaluate the significance of a low level finding

MOLECULAR PITFALLS - FALSE NEGATIVES

- Pre-analytic causes
 - DNA or RNA degradation or inadequate extraction
 - Higher false negatives for DNA testing from paraffin
 - DNA destruction by fixation, especially B5 or Bouin's fixatives
 - These false negatives can be detected, in part, by amplification of internal control genes

MOLECULAR PITFALLS - FALSE NEGATIVES

- Technical causes
 - Inadequate PCR conditions
 - Conditions of the test should be resolved during test development
 - Consensus primers for antigen receptor assays
 - Because these are “best fit” primers, usually for only the most commonly rearranged areas, they will miss a significant percentage of cases
 - Addition of primer directed against less commonly rearranged areas of the gene will often increase the detection rate
 - Consensus primers may not detect a rearrangement when a large background population of reactive cells is present

MOLECULAR PITFALLS - FALSE NEGATIVES

- Biologic causes
 - Incomplete VDJ rearrangement
 - Particularly in lymphoblastic neoplasms
 - IgH gene deletions and somatic hypermutation resulting in loss of primer annealing sites
 - Most common in follicular lymphomas and plasma cell dyscrasias
 - Second line testing, such as addition FRI or II region PCR, Igκ PCR or t(14;18) PCR may allow for detection of the clone
 - Alternate breakpoints not detected by primers
 - Common with DNA PCR, especially t(11;14), t(2;5) and t(14;18)

MOLECULAR PITFALLS - FALSE NEGATIVES

A NEGATIVE PCR RESULT SHOULD NEVER BE INTERPRETED AS SUFFICIENT EVIDENCE TO EXCLUDE A CLONAL POPULATION, ESPECIALLY A NEGATIVE IgH PCR TEST

PATENT ISSUES FOR GENE REARRANGEMENTS

- **PCR-Testing of the Immunoglobulin and T cell Receptor Loci:** *InVivoScribe* Technologies holds world-wide exclusive rights to the following patents for the identification and monitoring of leukemias, lymphomas, and other lymphoproliferative diseases:

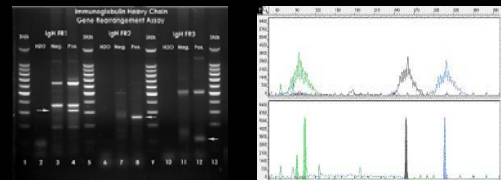
United States Patents Numbered 5,296,351 5,418,134
 Australian Patent Number 626,601
 Japanese Patent Number 2,781,438

NOTE: In the United States, Australia and Japan, sublicenses to our [*InVivoScribe*'s] patents are required for PCR testing of the immunoglobulin and T cell receptor loci.

FUTURE DIRECTIONS (1)

- Commercial kit assays
 - T and B cell gene rearrangements, and the most common translocations or mutations
- Currently available
 - *InVivoScribe* Technologies
 - Biomed-2

PATENT ISSUES FOR GENE REARRANGEMENTS



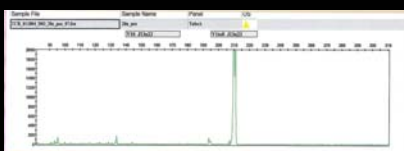
From <http://www.invivoscribe.com>

TCR GAMMA BIOMED-2 PCR

Reactive



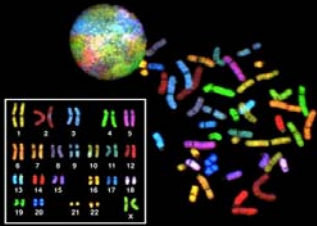
Clonal



FUTURE DIRECTIONS (2)

- Quantitative PCR
 - “Real time” results
 - Quantitation is useful in monitoring response to therapy and early molecular relapse
 - Already available in many diagnostic laboratories
- Spectral karyotyping (SKY)
 - Multicolor FISH analysis
 - A useful supplement to karyotype analysis
 - Helpful in detecting cryptic structural aberrations

SPECTRAL KARYOTYPE

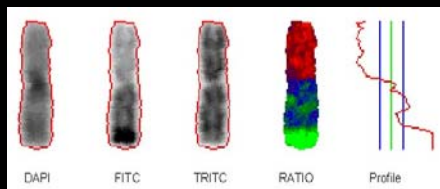


From National Genome Research Institute

FUTURE DIRECTIONS (3)

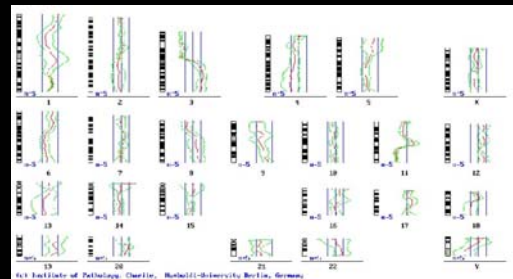
- Immunohistochemistry
 - Development of additional protein expression tests as cost effective surrogates for molecular changes (i.e. cyclin D1 for t(11;14) and ALK-1 for t(2;5))
- Comparative genomic hybridization (CGH)
 - Largely experimental method of comparing tumor DNA to normal DNA to identify areas of abnormalities that may not be visible by cytogenetic banding techniques

COMPARATIVE GENOMIC HYBRIDIZATION (CGH)



From Institute of Pathology, Humbolt University, Berlin

COMPARATIVE GENOMIC HYBRIDIZATION (CGH)

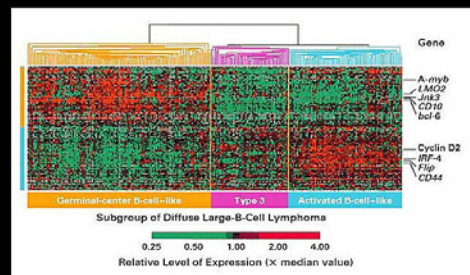


From Institute of Pathology, Charité - Humbolt-University Berlin, Germany

FUTURE DIRECTIONS (4)

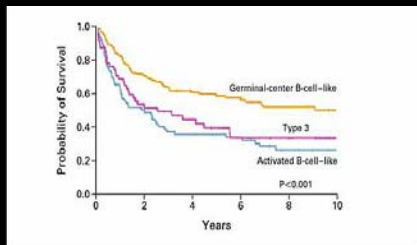
- Gene arrays
 - Allows for evaluation of thousands of genes for a given case
 - Generates enormous data for interpretation
 - Not ready for diagnostic use
 - Future development of prognostically significant, disease specific arrays may become common

GENE ARRAYS IN LARGE CELL LYMPHOMA



Rosenwald et al. NEJM 346:1937, 2002

GENE ARRAYS IN LARGE CELL LYMPHOMA



Rosenwald et al. NEJM 346:1937, 2002

GENE ARRAYS IN ADULT AML



GENERAL GUIDELINES

- Do not over-order
 - What is the question that you want to answer?
- Select the appropriate methodology for the question raised and for the material available
- Reconcile discrepancies with other tests before reporting results

OBJECTIVES

- Summarize the biology and physiology of some leukemia and lymphoma molecular mechanisms
 - Gene rearrangements and translocations
- Overview molecular tools and their rational use in hematopathology diagnosis
 - Southern blot, PCR, RT-PCR, Q-PCR, FISH

OBJECTIVES

- Illustrate pitfalls of molecular assays
 - False negatives and false positives
- Emphasize the need to integrate these data with other pathologic studies
 - Morphology and immunophenotyping
- Give a brief view of the future of molecular diagnostics in hematopathology
 - SKY, CGH, microarrays