

# **CASES DIFFICULT TO CATEGORIZE**

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# CASES DIFFICULT TO CATEGORIZE

- **Single defective rearrangement**
  - IGHV pseudogene
  - out-of-frame junction
  - stop codon(s)
  - absent HCDR3 “boundaries”
- **Double rearrangements**
  - double in-frame rearrangements with discordant mutation status
  - unmutated in-frame coexisting with a mutated out-of-frame rearrangement
- **Other ?**

# PSEUDOGENES

The image shows a screenshot of the NCBI PubMed website. The browser's address bar displays "Entrez PubMed". The page header includes the NCBI logo, the PubMed logo, and the text "A service of the National Library of Medicine and the National Institutes of Health". A "My NCBI" box with "Sign In" and "Register" links is in the top right. A navigation bar lists various database categories: All Databases, PubMed, Nucleotide, Protein, Genome, Structure, OMIM, PMC, Journals, and Books. The search bar contains the text "PubMed" in a dropdown menu, followed by "for CLL IMMUNOGLOBULIN PSEUDOGENE". There are "Go", "Clear", and "Save Search" buttons. Below the search bar are buttons for "Limits", "Preview/Index", "History", "Clipboard", and "Details". A pink highlighted message states "See [Details](#). No items found." The left sidebar contains links for "About Entrez", "Text Version", "Entrez PubMed", "Overview", "Help | FAQ", and "Tutorials".

# IGHV PSEUDOGENES IN CLL

- Infrequent
- Higher frequency on gDNA-PCR vs. RT-PCR
- Always (?) co-amplified with a rearrangement of a functional IGHV gene

# 1. Alignment for V-GENE and allele identification

Closest V-REGIONS (evaluated from the V-REGION first nucleotide to the 2nd-CYS codon)

	Score	Identity
<a href="#">Z12356</a> IGHV3-25*03 (P)	1399	98.61% (284/288 nt)
<a href="#">M99675</a> IGHV3-48*01	994	82.98% (239/288 nt)
<a href="#">Z12358</a> IGHV3-48*03	994	82.98% (239/288 nt)
<a href="#">AJ879486</a> IGHV3-23*04	985	82.63% (238/288 nt)
<a href="#">AB019438</a> IGHV3-48*02	985	82.63% (238/288 nt)

EMQLVESGG.GLAKPAWSPRLSCAASQFTFSSYY . . . .MNCVRQAPGNGLLELV\*QVNPNGGS  
T . . YLIDSGK.DRFNTRS RNAKNTLHLQMN SLKTEDTALFYCTSL\*GPLTISGDLTFDPW

Elke Boone, H-Hartziekenhuis Roeselare, Belgium

Molecule type: cDNA

Single amplified rearrangement

**ERIC DATABASE OF PROBLEMATIC CASES**

# What to say? What to do?

- For the moment, the clinical correlation cannot be defined.
- A definitive result should not be given to clinicians.
- Repeat analysis on cDNA from a new blood sample
- Alternative primers (Ghia P et al. Leukemia 2007;21:1-3)

# CASES DIFFICULT TO CATEGORIZE

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## 4. Results of IMGT/JunctionAnalysis

Maximum number of accepted mutations in: 3'V-REGION = 0, D-REGION = 2, 5'J-REGION = 0

### Analysis of the JUNCTION

D-REGION is in reading frame 3.

Click on mutated (underlined) nucleotide to see the original one:

Input	V name	3'V-REGION	N1	D-REGION	N2	5'J-REGION	J name	D name	Vmut	Dmut	Jmut	Ngc
1	<a href="#">IGHV5-51*01</a>	tgtgcgagaca	tggaggacct	<u>.ggatattgtagtggtgtagctgcta....</u>	tgg	.....actacggtatggacgtctgg	<a href="#">IGHJ6*02</a>	<a href="#">IGHD2-15*01</a>	0	0	0	8/13

### Translation of the JUNCTION

Click on mutated (underlined) amino acid to see the original one:

	104	105	106	107	108	109	110	111	111.1	111.2	111.3	111.4	112.5	112.4	112.3	112.2	112.1	112	113	114	115	116	117	118	Frame	CDR3	IMGT length	Molecular mass	pI
	C	A	R	R	G	G	P	G	Y	C	S	G	G	S	C	Y	#	#	Y	G	M	D	V	W	-	22	2,326.6	7.23	
1	tgt	gcg	aga	cat	gga	gga	cct	gga	tat	tgt	agt	ggt	ggt	agc	tgc	tat	gg.	.ac	tac	ggt	atg	gac	gtc	tgg	-	22	2,326.6	7.23	

Molecule type: cDNA  
 Single amplified rearrangement

★ ★ IMGT/V-QUEST

## 1. Alignment for V-GENE and allele identification

Closest V-REGIONS (evaluated from the V-REGION first nucleotide to the 2nd-CYS codon)

	Score	Identity
<a href="#">Z17392</a> IGHV3-74*02	1138	88.54% (255/288 nt)
<a href="#">L33851</a> IGHV3-74*01	1129	88.19% (254/288 nt)
<a href="#">J00239</a> IGHV3-74*03	1129	88.19% (254/288 nt)
<a href="#">M99675</a> IGHV3-48*01	994	82.98% (239/288 nt)
<a href="#">AJ879484</a> IGHV3-h*01 (P)	988	83.15% (237/285 nt)

### Analysis of the JUNCTION

D-REGION is in reading frame 3.

Click on mutated (underlined) nucleotide to see the original one:

Input	V name	3'V-REGION	N1	D-REGION	N2	5'J-REGION	J name	D name	Vmut	Dmut	Jmut	Ngc
ITA-Mir	<a href="#">IGHV3-74*02</a>	tgtgtaaga	ggcgccgtttgg	...tagtgggagct....	cttagagtggcctact	.....g	<a href="#">IGHJ4*03</a>	<a href="#">IGHD1-26*01</a>	1	0	0	17/28

### Translation of the JUNCTION

Click on mutated (underlined) amino acid to see the original one:

104	105	106	107	108	109	110	111	111.1	112.1	112	113	114	115	116	117	118	Frame	CDR3	Molecular mass	pI	
C	Y	R	G	A	V	W	*	W	E	L	L	E	W	P	T	#		length			
ITA-Mir	tgt	gta	aga	ggc	gcc	ggt	tgg	tag	tgg	gag	ctc	tta	gag	tgg	cct	act	..g	-	15	1,845.16	4.4

EVQLVESGG.G\*VQPGGSLRLYCTASGFATSNHW....MSWVRQVSGRGLV\*VSRFNSN  
 GRTK..TYADSVK.GRFTISRDNGKNMLYLQMNSLRPEDTALYDCVRGAVW\*W

Molecule type: cDNA  
 Single amplified rearrangement

# TRANSCRIBED OUT-OF-FRAME

## 70/11314 LIGM-DB sequences

CASE	IGHV	CDR3 AMINO ACIDS	ENTITY
AJ008173	IGHV3-33*01	CARWL*QKWLALGTSITMDVW	Sjogren
AJ234284	IGHV4-34*02	CARGGVTVPPIPKISDLF#SW	Follicular Lymphoma
AJ300792	IGHV4-4*02	CARLGITGTSD#FEIW	Kawasaki disease
AJ414019	IGHV4-b*01	CARGIRTYYDFWSGYYTGPPFR#WFDSW	CLL
AJ496483	IGHV3-23*01	CARDLYCTTTSC#W	Rheumatoid arthritis
AJ556739	IGHV3-23*01	CA*GMGGFYDIW	Multiple sclerosis
AJ579123	IGHV4-4*07	CARDRIVVVVAAT#YFHHW	Normal tonsillar cell
AY003832	IGHV1-69*01	CARDV*DLVVVVAAPST##W	Normal plasma cell
AY640477	IGHV3-30*03	CVKDSSSSTLY#YFDLW	Allergic rhinitis
L28051	IGHV3-21*01	CARLRHH*TKP##FDYW	SLE
X98943	IGHV1-3*01	CAKRQENTFFSGMDV#W	Hashimoto's thyroiditis
Z74669	IGHV4-59*01	CARDFPYCGGDCYSAMDYYDSSGYHSLIS*L#W	Burkitt's Lymphoma

# SINGLE OUT-OF-FRAME



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# STOP CODONS IN HCDR3

## “INTERNAL” CLL IGHV-D-J SEQUENCES

CASE	IGHV	HCDR3 AMINO ACID SEQUENCE
Swe-393-I	IGHV3-30*03	CV*LSERGGVLWD
Swe-237-II	IGHV3-9*01	CAKDRQYCSSTSCYTVFP*DFWSGYLFMGDYYYYYMDVW
Swe-308-I	IGHV1-69*01	CAISPSQP*QWLALYYYYGMDVW
P795	IGHV3-74*02	CARDPPRLL***WLQRLR*DYW
FRA-154	IGHV3-49*03	CKFPL*YIWSHASHGTPQRGARNYNYYYGMDVW
P4392	IGHV3-30*03	CAKDQGPPLWW*LLQGASVDAFDIW
N2526	IGHV4-28*01	CARVLGPSKYTGRKE*FSLGL**YQLLPSGLYYYYGMDVW

# STOP CODONS IN HFR1-HFR3

## “INTERNAL” CLL IGHV-D-J SEQUENCES

IGHV3-33\*01 GRFTISRDN SKNTLYLQMN SLRAEDTAVYYC (FR3 74-104)

P130 -----T-----D-----T--\*-

IGHV3-74\*01 GLVQPGGSLRLSCAASGFTFSSYW...MHWVRQAPGKGLVWVSR (FR1-11/FR2-55)

ITA-Mir -\*-----YC-A---A--NH-....-----VS-R---\*---

IGHV4-34\*01 SRVTISVDTSKNQFSLKLSSVTAADTAVYYC (FR3 74-104)

P3504 -----\*-----T-----

\* STOP CODON

# **Nonsense-mediated mRNA decay**

## **An mRNA surveillance mechanism**

**mRNAs carrying a premature termination codon are highly unstable.**

**Nonsense-mediated mRNA decay (NMD) recognizes these mRNAs and degrades them.**

**Analysis of the well characterized human genes in RefSeq reveals that the vast majority are not candidates for NMD.**

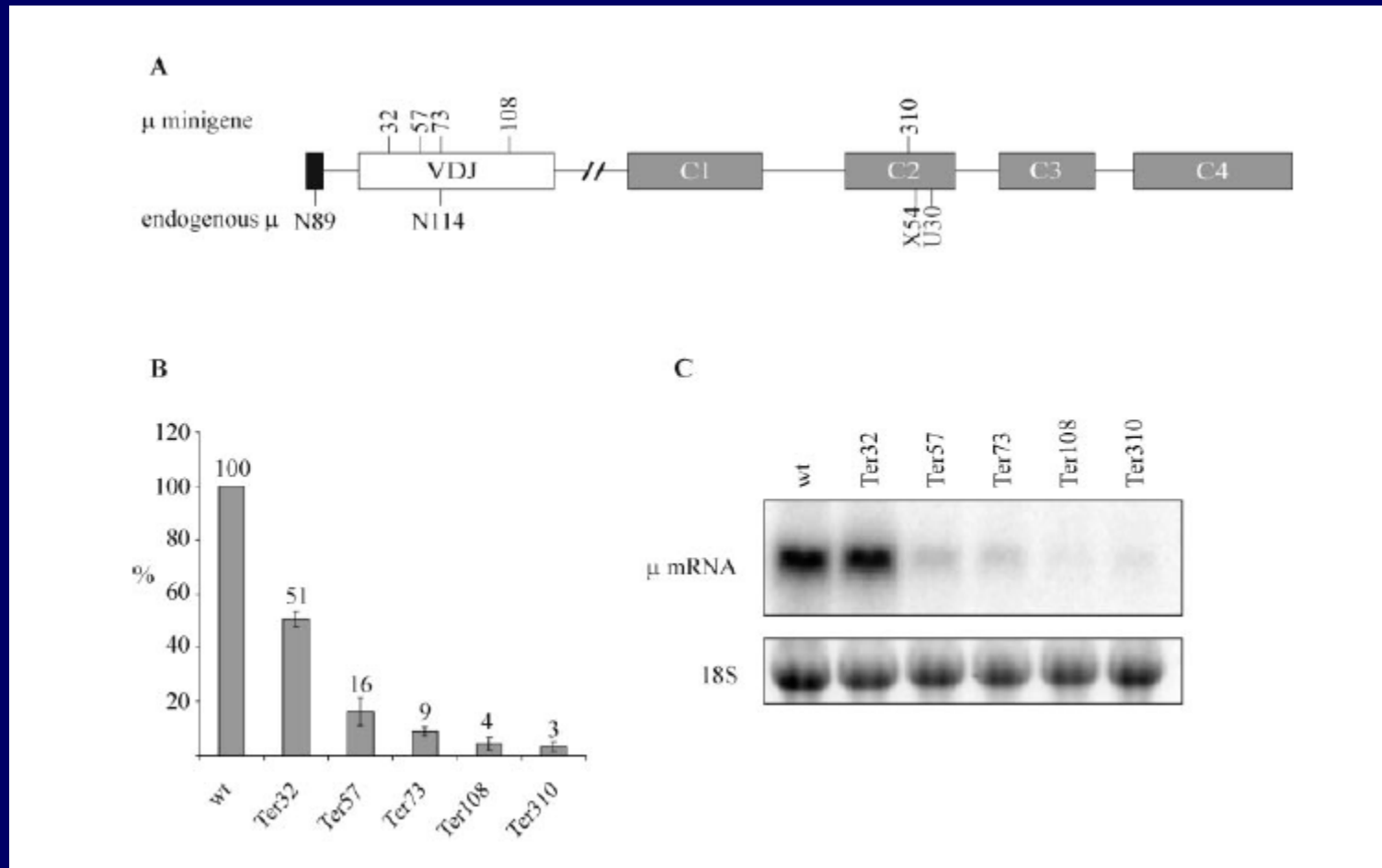
3304–3315 *Nucleic Acids Research*, 2004, Vol. 32, 11  
DOI: 10.1093/nar/gkh651

## Efficient downregulation of immunoglobulin $\mu$ mRNA with premature translation-termination codons requires the 5'-half of the VDJ exon

Marc Bühler, Alexandra Paillusson and Oliver Mühlemann\*

**NMD appears to be significantly more efficient for mRNAs of genes belonging to the IG superfamily**, which frequently acquire premature termination codons during VDJ rearrangement and SHM, than for mRNAs of other genes.

It is conceivable that specific signals might have evolved in **genes of the IG superfamily** that trigger a **particularly efficient mode of NMD to avoid production of truncated TCR and immunoglobulin polypeptide chains.**



...Most interestingly, the **efficiency of NMD** depends on the position of the PTC and **increases as the PTC is moved further downstream in the Ig-m gene**, as reflected by the decreasing PTC+ mRNA levels...

# Defective IG transcripts

## *Failure of NMD???*

*Benito C et al. Transcript expression of two Ig $\lambda$  rearrangements and RAG-1/RAG-2 in a mature human B cell producing IgM $\lambda$  **islet cell autoantibody**. J Clin Immunol 2003. 23: 107*

*Darlow et al. Non-functional immunoglobulin G transcripts in a case of **hyper-immunoglobulin M syndrome** similar to type 4. Immunology 2004. 111: 212-222*

# Defective IGKV-J transcripts

260 CLL patients

**14 defective IGKV-J transcripts**

(out of frame, stop codons, insertions-deletions)

Belessi C et al. Analysis of expressed and non-expressed IGK locus rearrangements in **chronic lymphocytic leukemia**. Mol Med 2005;11(1-12):52-8.

# What to say? What to do?

- For the moment, the clinical correlation cannot be defined.
- A definitive result should not be given to clinicians.
- Repeat analysis?
- Alternative primers? (Ghia P et al. Leukemia 2007;21:1-3)

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# Absent CDR3 “landmarks”

- Excessive base trimming by exonuclease activity
- Somatic mutation

### Analysis of the JUNCTION

D-REGION is in reading frame 1.

Click on mutated (underlined) nucleotide to see the original one:

Input	V name	3'V-REGION	N1	D-REGION	N2	5'J-REGION	J name	D name	Vmut	Dmut	D
AJ239361 HSA239361 H...	<u>IGHV3-30-3*01</u>	tgtgtgaga	ccccggg	gggtatagca <u>aac</u> actg....	cccaaaagagtcgactagt	.....g	<u>IGHJ6*01</u>	<u>IGHD6-13*01</u>	1	2	

### Translation of the JUNCTION

Click on mutated (underlined) amino acid to see the original one:

104	105	106	107	108	109	110	111	111.1	112.1	112	113	114	115	116	117	118	Frame	CDR3 - IMGT length	Molecular mass	pI
C	<u>V</u>	R	P	G	G	Y	S	<u>N</u>	<u>N</u>	C	P	K	S	R	L	V	+	15	1,850.16	10.03
AJ239361 HSA239361 H...	tgt	<u>gtg</u>	aga	ccc	ggg	ggg	tat	agc	<u>aac</u>	<u>aac</u>	tgc	cca	aag	agt	cga	cta	gtg			

# MUTATED C-104

## 29/11314 LIGM-DB sequences

CASE	IGHV	IGHD	IGHJ	CDR3 AMINO ACIDS
AB063774	IGHV1-69*01	IGHD1-26*01	IGHJ6*02	FARDLGGTTGWGQVYSNGMDVW
AB063778	IGHV1-69*01	IGHD1-26*01	IGHJ6*02	WARDLGATTGWGQKYYNGMDVW
AJ413992	IGHV3-7*01	IGHD3-3*01	IGHJ4*02	SARDLTIFGSGYLDYW
AJ415267	IGHV3-30*03	IGHD3-9*01	IGHJ4*03	WARGLLTVNFEPVYFDSW
AY003820	IGHV3-23*01	IGHD7-27*01	IGHJ4*02	WAKDRWGDYYPYW
AJ239374	IGHV3-73*01		IGHJ4*02	FIRHSL
U70088	IGHV3-30*03	IGHD6-6*01	IGHJ4*02	RAKGLAKYSSSSLDW
X87054	IGHV3-23*01	IGHD3-10*01	IGHJ3*02	SAKGSASGNPYKAFDIW
Z68415	IGHV3-30-3*01	IGHD3-3*01	IGHJ3*02	SARDDLGLTIFGVKGSW

CLL

# MUTATED W-118

## 30/11314 LIGM-DB sequences

CASE	IGHV	IGHD	IGHJ	CDR3 AMINOACIDS
AF006519	IGHV1-46*01	IGHD4-17*01	IGHJ4*02	CATDYSKNSTRMPTFLDFRGQG
Z74682	IGHV4-59*01	IGHD6-13*01	IGHJ3*02	CVRGGTQPFDIRGQG
AF103213	IGHV3-23*01	IGHD5-24*01	IGHJ4*02	CAKDSFSYFDFGGQG
AF381608	IGHV4-39*01	IGHD6-19*01	IGHJ5*02	CATHQWLEGGGLWFDSLGG
AJ239361	IGHV3-30-3*01			CVRPGGYSNNCPKSRLVGG
AY927668	IGHV1-69*01		IGHJ4*02	CARGPDTGGYYYFYGGQG
Z36886	IGHV3-73*02		IGHJ5*02	CASGSYLKGG
Z98737	IGHV3-15*07		IGHJ3*01	CTTGGTVGALGG
AJ239389	IGHV3-7*01	IGHD2-15*01	IGHJ3*02	CARVADKGYCRGASCYGRSGAFDICGG
AJ245293	IGHV3-30*09	IGHD6-13*01	IGHJ4*02	CARAIAAAGRVFDCGG
AJ279518	IGHV4-31*03	IGHD3-16*02	IGHJ4*02	CARDRLWEMATILVGG
AY393112	IGHV1-46*01	IGHD3-10*02	IGHJ4*02	CARVGAAADE*GG

CLL

# What to say? What to do?

A JUNCTION will extend from 2nd-CYS 104 to J-TRP included.

J-TRP is easily identified when the conserved motif

**Trp-Gly-X-Gly**

of the J-REGION is present.

[IMGT/V-QUEST Documentation](#)

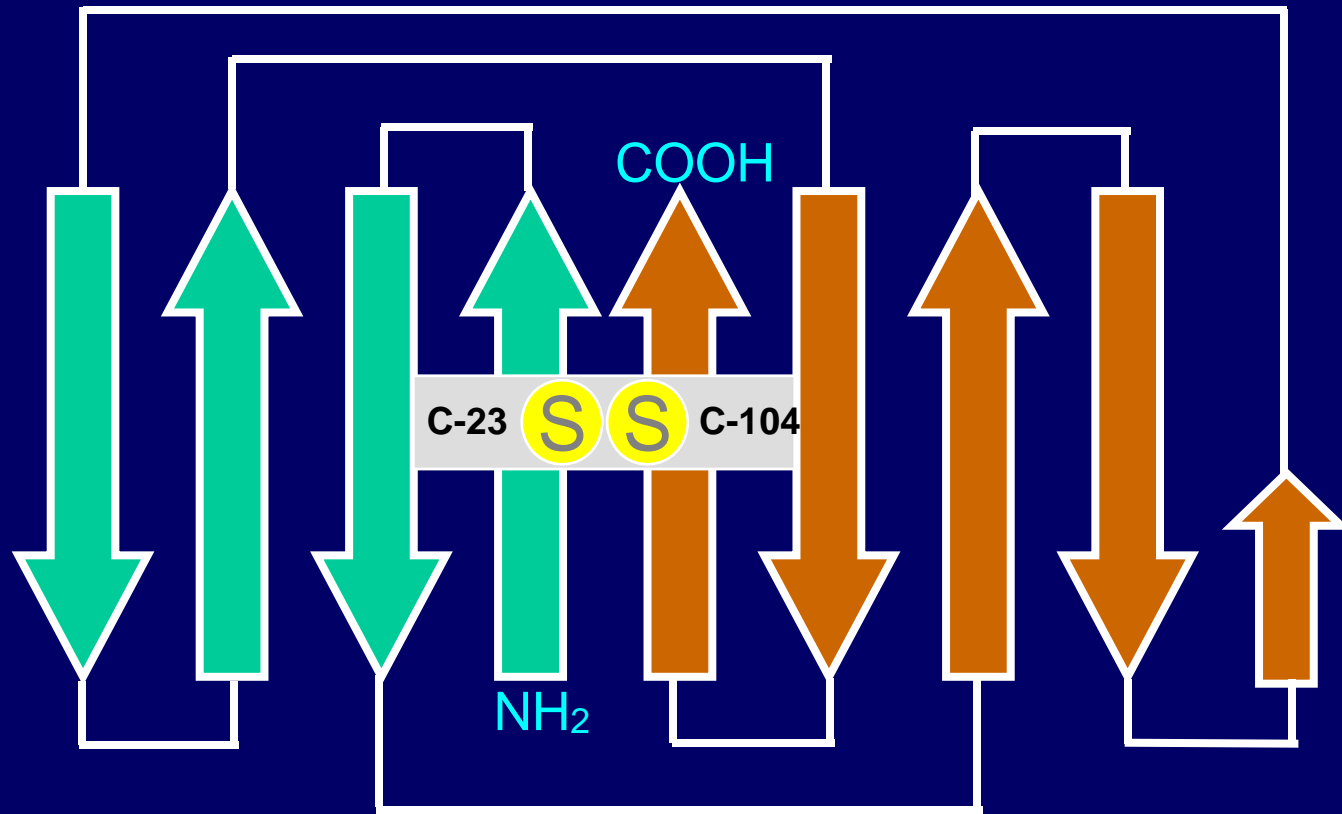
ImMunoGeneTics Information system

<http://imgt.cines.fr>

Citing IMGT/V-QUEST: Giudicelli, V. et al. Nucl. Acids Res. 2004, 32, W435-440 [PMID\\_15215425](#)

You are in the documentation of the new IMGT/V-QUEST, upgraded for multiple sequences and with new functionalities. **NEW!**

# What to say? What to do?



**What to say? What to do?**



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- **Other ?**

# DOUBLE IN-FRAME REARRANGEMENTS

- Relative frequency within cohorts: varies
  - France  $3/763$  (0.4%)
  - Greece (mainly cDNA)  $13/581$  (2.2%)
  - Italy  $0/244$  (0.0%)
  - Sweden (mainly gDNA)  $20/503$  (3.9%)

Overall frequency: 1.7% (36/2091 cases)

# DOUBLE IN-FRAME REARRANGEMENTS

## Interpretation???

Productive (in-frame) IG rearrangements are not always transcribed.

*Belessi C et al. Analysis of expressed and non-expressed IGK locus rearrangements in chronic lymphocytic leukemia. Mol Med 2005;11(1-12):52-8.*

Biclonal population?

*Sanchez ML et al. Incidence and clinicobiologic characteristics of leukemic B-cell chronic lymphoproliferative disorders with more than one B-cell clone. Blood. 2003;102:2994-3002*

# DOUBLE IN-FRAME REARRANGEMENTS

Interpretation???

**Lack of allelic exclusion (allelic inclusion)**

- Expression of multiple antigen receptors
- Also called “receptor dilution”

# DOUBLE IN-FRAME REARRANGEMENTS

## Lack of allelic exclusion (allelic inclusion)

- Often employed by autoreactive B cells
  - Kenny JJ, Rezanka LJ, Lustig A, et al. Autoreactive B cells escape clonal deletion by expressing multiple antigen receptors. *J. Immunol.* 2000; 164: 4111-4119
- Frequent feature in the normal marginal-zone
  - Li Y, Li H, Weigert M. Autoreactive B cells in the marginal zone that express dual receptors. *J. Exp. Med.* 2002; 195: 181-188
- Previously reported in CLL, at least at transcript level
  - Rassenti LZ, Kipps TJ. Lack of allelic exclusion in B cell chronic lymphocytic leukemia. *J. Exp. Med.* 1997; 185: 1435-1445

# DOUBLE IN-FRAME WITH DISCORDANT MUTATION STATUS

14/36 cases

CASE	IGHV	%	CASE	IGHV	%
FAV-90	IGHV4-34	100	Swe-186-I	IGHV3-33	99,6
FAV-90b	IGHV3-23	94,8	Swe-186-II	IGHV4-59	94,3
N1182A	IGHV3-66	98,3	Swe-198-I	IGHV3-64	98,8
N1182B	IGHV3-48	87,6	Swe-198-II	IGHV6-1	88,8
P1346A	IGHV3-48	100	Swe-293-I	IGHV4-b	86,8
P1346B	IGHV4-61	92,1	Swe-293-II	IGHV3-33	100
P1402A	IGHV2-5	87	Swe-439-I	IGHV4-34	100
P1402B	IGHV3-72	98,3	Swe-439-II	IGHV3-9	96,5
P532A	IGHV1-58	96,6	Swe-448-I	IGHV3-74	96,1
P532B	IGHV1-69	99,7	Swe-448-II	IGHV1-69	100
P573A	IGHV3-7	93,5	Swe-485-I	IGHV1-69	100
P573B	IGHV1-69	100	Swe-485-II	IGHV2-70	97,6
Swe-158-I	IGHV2-70	95,2	Swe-497-I	IGHV4-61	98,2
Swe-158-III	IGHV3-64	99,1	Swe-497-II	IGHV4-61	94,2

# DOUBLE IN-FRAME REARRANGEMENTS

**Biclonal population? What to do?**

Re-check flow results

Two IGH sequences obtained using gDNA?

Repeat analysis using cDNA

One or two light chains?

Perform RT-PCR for light chain genes

# DOUBLE IN-FRAME REARRANGEMENTS

Discordant mutation status

What to say? What to do?



# DOUBLE IN-FRAME REARRANGEMENTS

## Discordant mutation status

Light chains may offer helpful hints

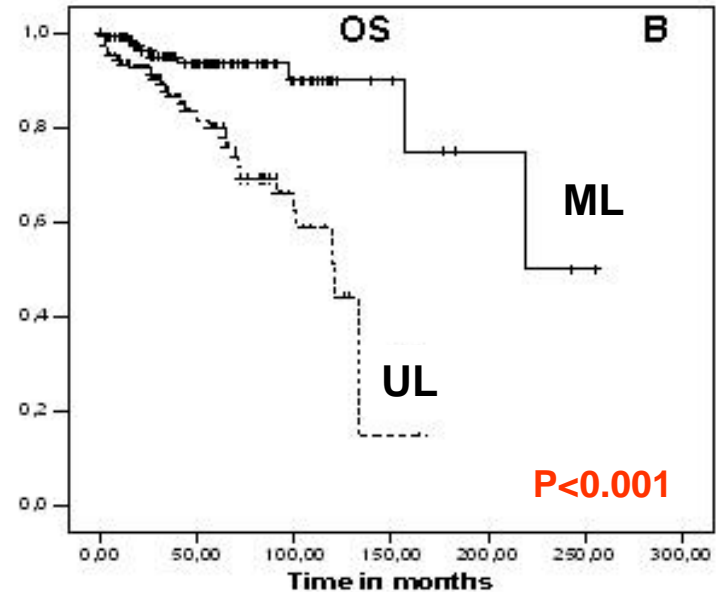
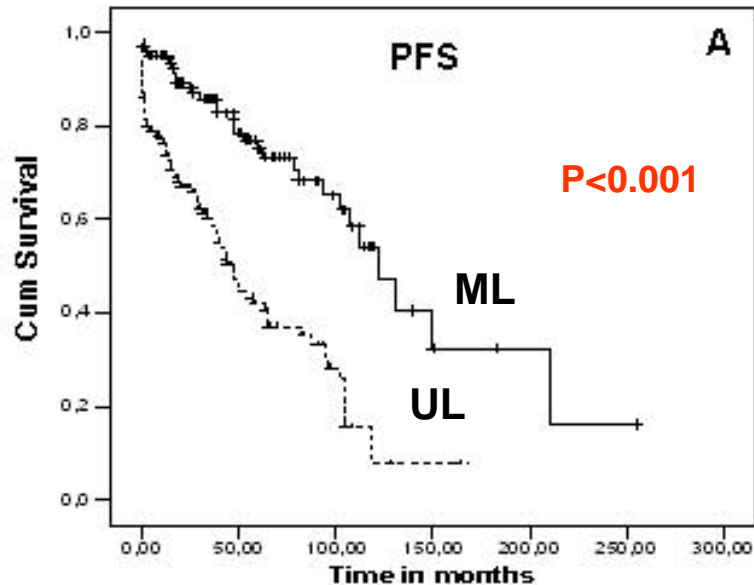
CASE	IGHV	%	IGK/LV	%	CD38	ZAP70	Genetics	Current status
N1182A	IGHV3-48	87,6	IGKV3-20	96,2	1	ND	ND	Alive, at 60 mo
N1182B	IGHV3-66	98,3						
P1346A	IGHV3-48	100	IGLV1-44	100	1,7	27	Trisomy 5	Dead, at 31 mo
P1346B	IGHV4-61	92,1						
P1402A	IGHV2-5	87,0	IGLV9-49	95,2	1,8	5	del13q	Alive, at 42 mo
P1402B	IGHV3-72	98,3						
P532A	IGHV1-58	96,6	IGKV1-39	97,6	28,7	ND	ND	Alive, at 55 mo
P532B	IGHV1-69	99,7						
P573A	IGHV3-7	93,5	IGKV3-20	96,8	3,4	7,6	Complex	Alive, at 106 mo
P573B	IGHV1-69	100						

# DISCORDANT IG HEAVY/LIGHT CHAIN MUTATION STATUS IN CLL

Type of rearrangements	%
<b>CLL-kappa</b>	
IGKV-M + IGHV-M	46.9
IGKV-M + IGHV-U	3.4
IGKV-U + IGHV-U	37.7
IGKV-U + IGHV-M	12.0
<b>CLL-lambda</b>	
IGLV-M + IGHV-M	42.1
IGLV-M + IGHV-U	4.2
IGLV-U + IGHV-U	41.1
IGLV-U + IGHV-M	12.6

# **LIGHT CHAIN MUTATION STATUS IN CLL**

**PROGNOSTIC IMPLICATIONS?**



Multivariate Cox regression analysis revealed that only IGH mutation status and clinical stage remained statistically significant variables for both PFS and OS.

# DOUBLE IN-FRAME REARRANGEMENTS

Discordant mutation status

What to say? What to do?



# DOUBLE IN-FRAME REARRANGEMENTS

## Discordant mutation status

### What to say? What to do?

- Assess other prognostic indicators and use common sense.
- Be cautious.
- More cases are necessary to reach meaningful conclusions.

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- **Other ?**

# Unmutated in-frame coexisting with a mutated out-of-frame rearrangement

Very infrequent

CASE	IGHV	%	FRAME	
Swe-145-I	IGHV3-23	98,2	IF	“UM”
Swe-145-II	IGHV3-66	96,4	OF	
Swe-267-I	IGHV3-48	98,6	IF	“UM”
Swe-267-II	IGHV1-69	96,4	OF	

...as for any mathematical cutoff value applied to biological phenomenon, **one has to be cautious when dealing with ‘borderline cases’...**

# Unmutated in-frame coexisting with a mutated out-of-frame rearrangement

Very infrequent

CASE	IGHV	%	FRAME	
Swe-145-I	IGHV3-23	98,2	IF	UM?
Swe-145-II	IGHV3-66	96,4	OF	
Swe-267-I	IGHV3-48	98,6	IF	UM?
Swe-267-II	IGHV1-69	96,4	OF	

# “Bystander” mutagenesis

Both rearrangements are somatically mutated

**More mutated non-productive rearrangement**

**Somatic hypermutation without selection for expression of a functional antigen receptor**

**What to say? What to do?**



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  - absent HCDR3 “landmarks”
- **Double rearrangements**
  - double in-frame rearrangements with discordant mutation status
  - unmutated in-frame coexisting with a mutated out-of-frame rearrangement
- **Other ?**

**Other?**

**Please let us know!!!**

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