Analysis of HNPCC and BRCA mutations: screening of unclassified variants (UVs) for splicing defects

Mario Tosi

Inserm U614, Faculté de Médecine et de Pharmacie de Rouen

Institut Hospitalo-Universitaire de Recherche Biomédicale de Rouen

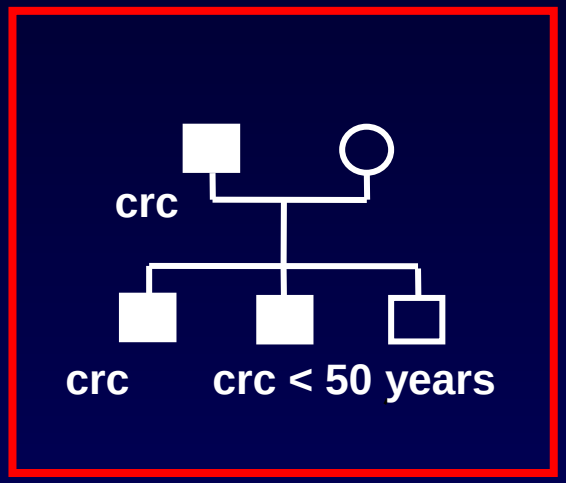
London, 3rd March 2008

Eurasnet workshop : RNA splicing and genetic diagnosis

Worshop organizers : Diana Baralle, Nick Cross

HNPCC (*Hereditary Non Polyposis Colorectal Cancer*) OR LYNCH SYNDROME

2 - 5 % of colorectal cancers

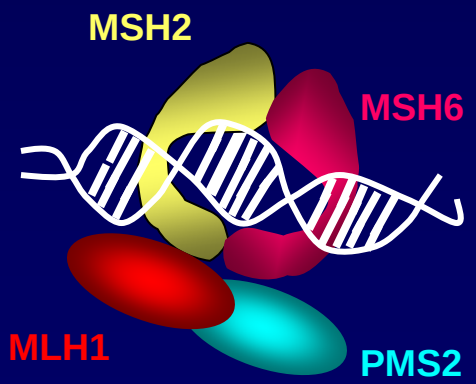


- Colon rectum +++
- Endometrium +++
- Small bowel
- Urinary tract
- Stomach
- Ovary
- Biliary tract

Autosomal dominant genetic predisposition

MLH1 (3p21)
MSH2 (2p22-p21)
MSH6 (2p16)
PMS2 (7p22)

MMR (mismatch repair) genes

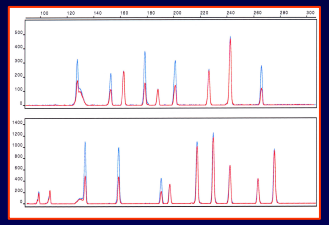


MMR GENE ALTERATIONS IN THE LYNCH SYNDROME

Data for families recruited with stringent criteria (Amsterdam)

-Pathogenic point mutations

MLH1	25 %
MSH2	25 %
MSH6, PMS2	5 %
- MSH2 gene rearrangements	10 %
- MLH1 rearrangements	< 4 %
	< 70 %



QMPSF

MLPA

+ Many variants of unknown significance (UVs)
Missense variants, silent (at the level of translation), intronic variants

Di Fiore et al., J Med Genet 2004, 41:18-20

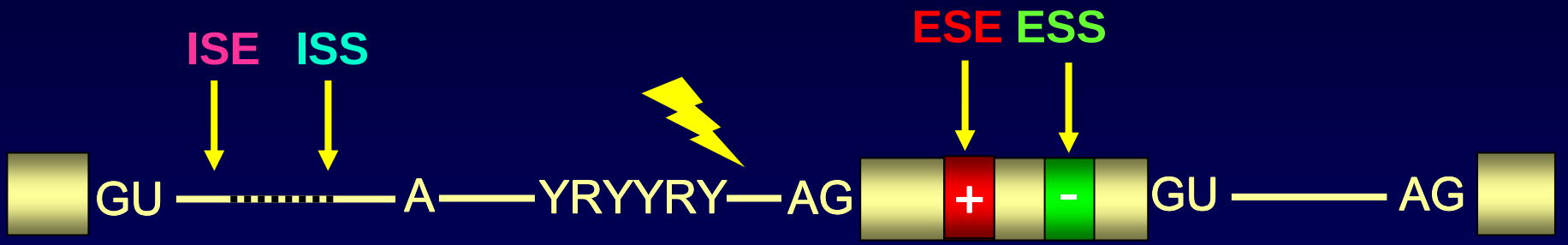
SPLICING REGULATORY ELEMENTS

ESE *Exonic Splicing Enhancer*

ESS *Exonic Splicing Silencer*

ISE *Intronic Splicing Enhancer*

ISS *Intronic Splicing Silencer*



LEVELS OF ANALYSIS

PATIENT RNA
in most cases not available

In silico predictions
not sufficient for diagnosis
not always accurate

Functional splicing assays
time-consuming but very useful

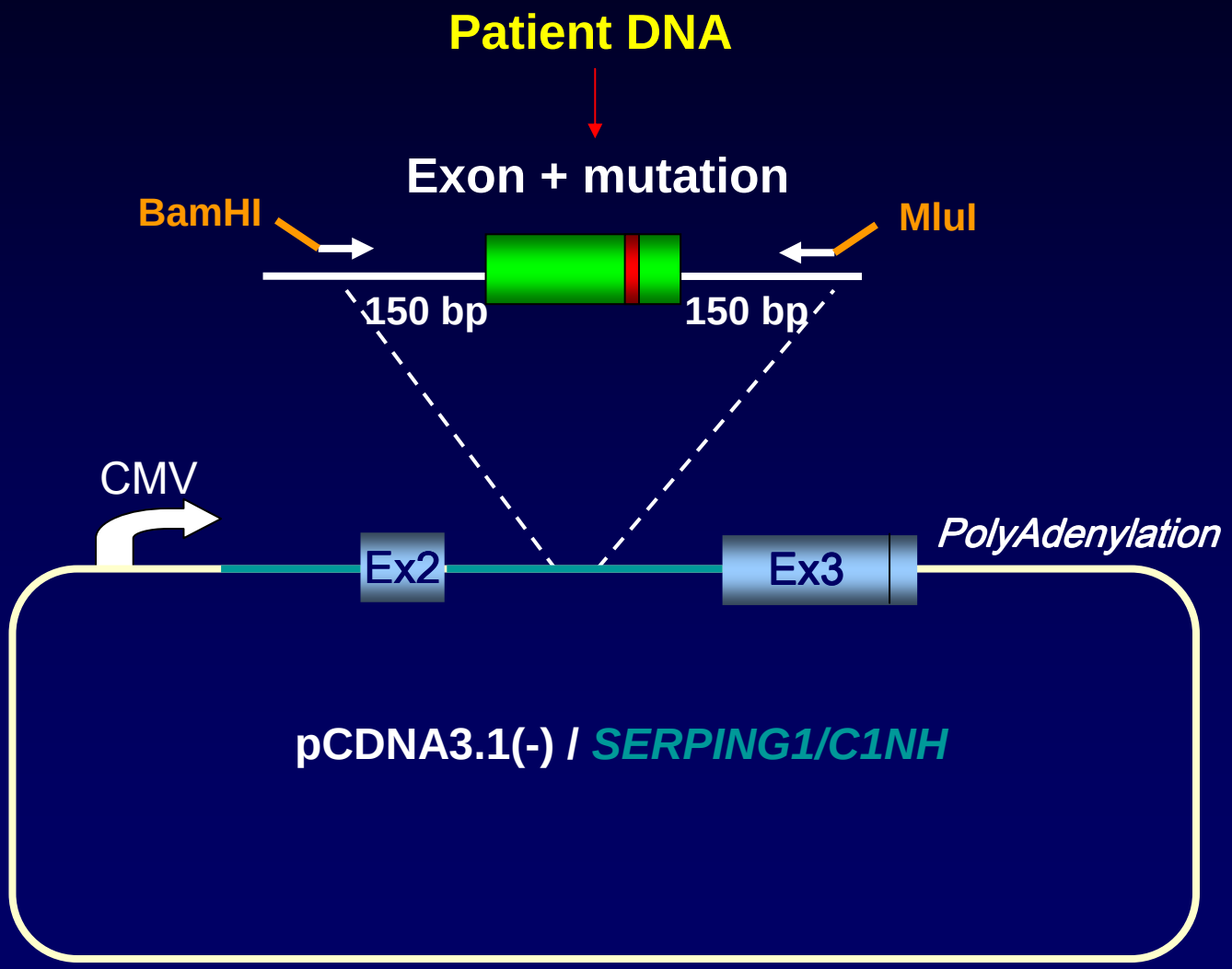
Splicing reporter minigene assays, reviewed in:

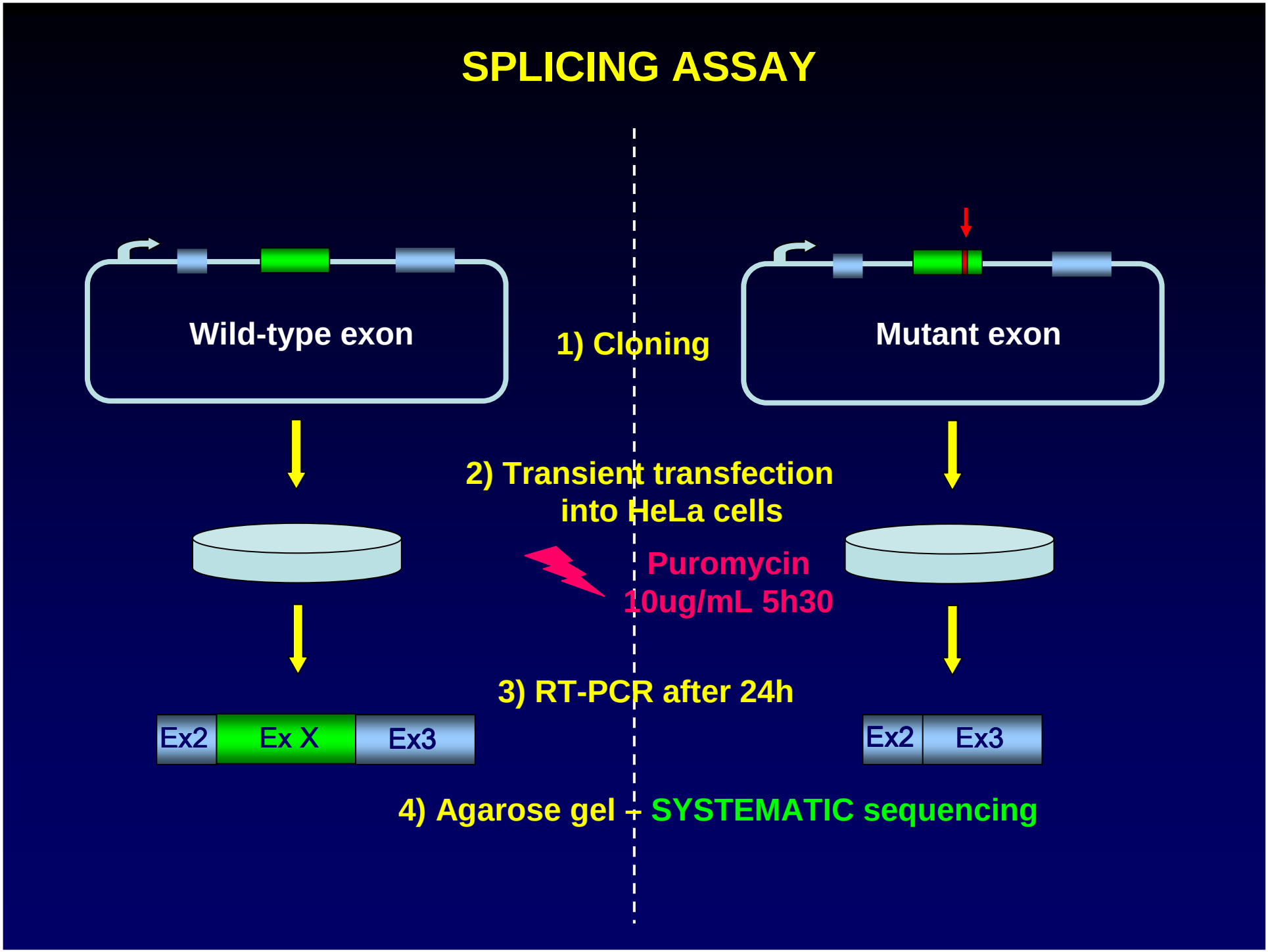
Baralle and Baralle *J. Med. Genet.* 2005;42;737-748

Cooper TA. Methods 2005; 37:331-340

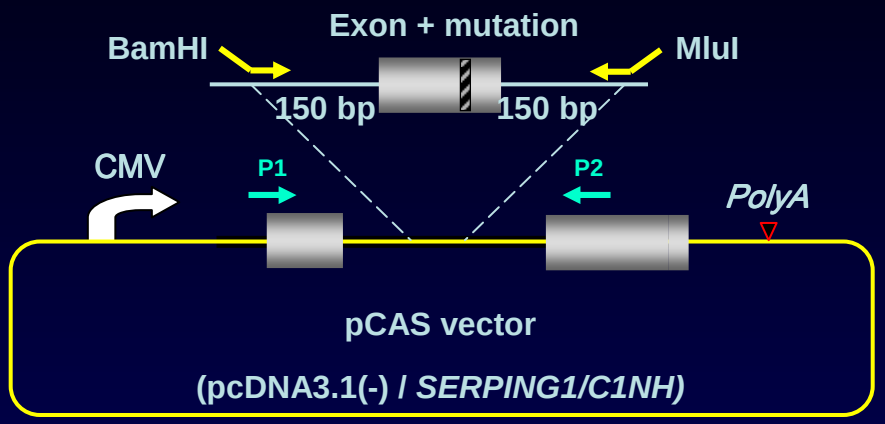
SPLICING ASSAY

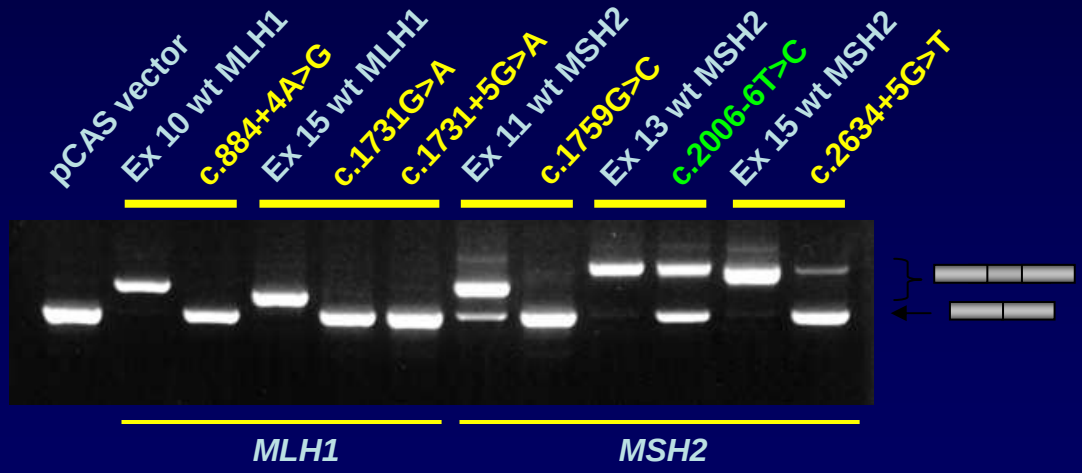
Patient blood samples (PaxGene) for RNA
often not available




SPLICING ASSAY
Wild-type exon
1) Cloning
Mutant exon
2) Transient transfection into HeLa cells
Puromycin 10ug/mL 5h30
3) RT-PCR after 24h
Ex2
Ex X
Ex3
Ex2
Ex3
4) Agarose gel – SYSTEMATIC sequencing

EXAMPLES OF VARIANTS THAT MODIFY OR GENERATE EXON BOUNDARIES





All RT-PCR products are sequenced

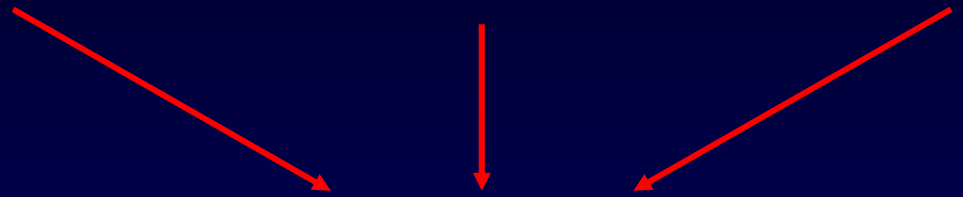
Tournier et al. (2008). Human Mutation, in press

MLH1, *MSH2* VARIANTS ANALYSED (FEB. 2008)

74 Missense mutations
5 single codon deletions
1 del/ins

16 Silent mutations

34 Intronic mutations other than conserved AG/GT



130 *MLH1* (73), *MSH2* (57) variants analysed

33 / 130 (25%) affect splicing

27 Alteration of splice sites, or
Generation of new splice sites, or
Activation of crytpic splice site

6 Mutations of putative splicing regulatory elements

MUTATIONS OF PUTATIVE SPLICING REGULATORY ELEMENTS

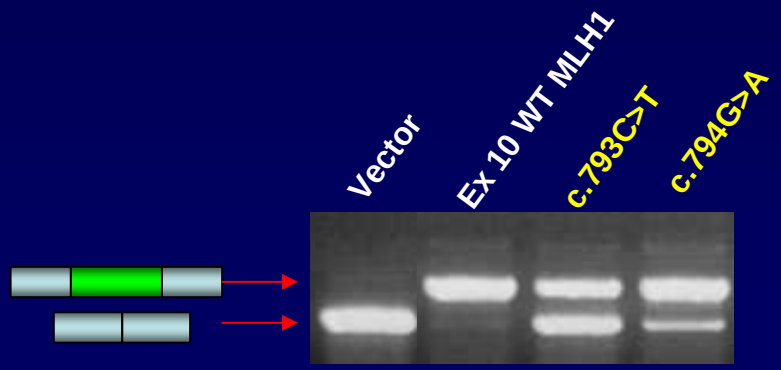
MLH1
Exon 10, 3rth exon position
4th exon position

In silico predictions

Variant	ESEfinder	RescueESE	PESX	ASSA
c.793C>T	-	-	-	-
c.794G>A	SC35, SRp40 abolished	-	-	SC35 ↓

pCAS splicing assay

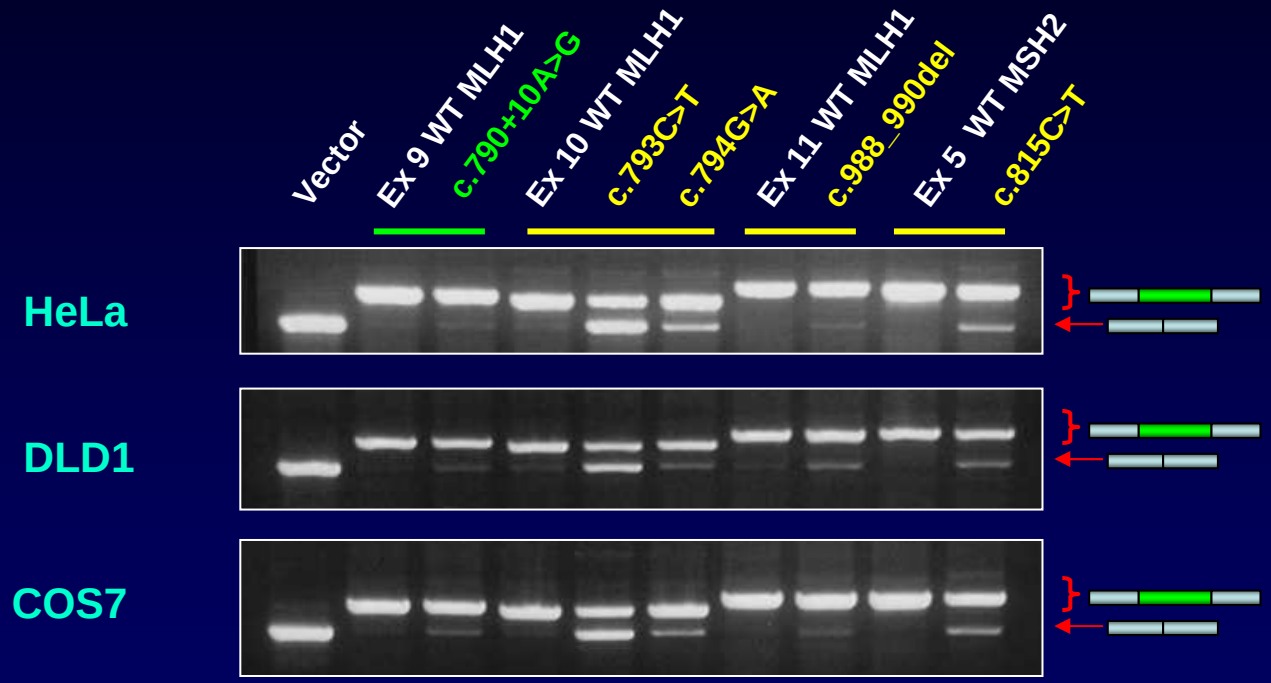
c.793C>T : partial exon 10 skipping +++
c.794G>A : partial exon 10 skipping ++



→ Identification of a new ESE element ?

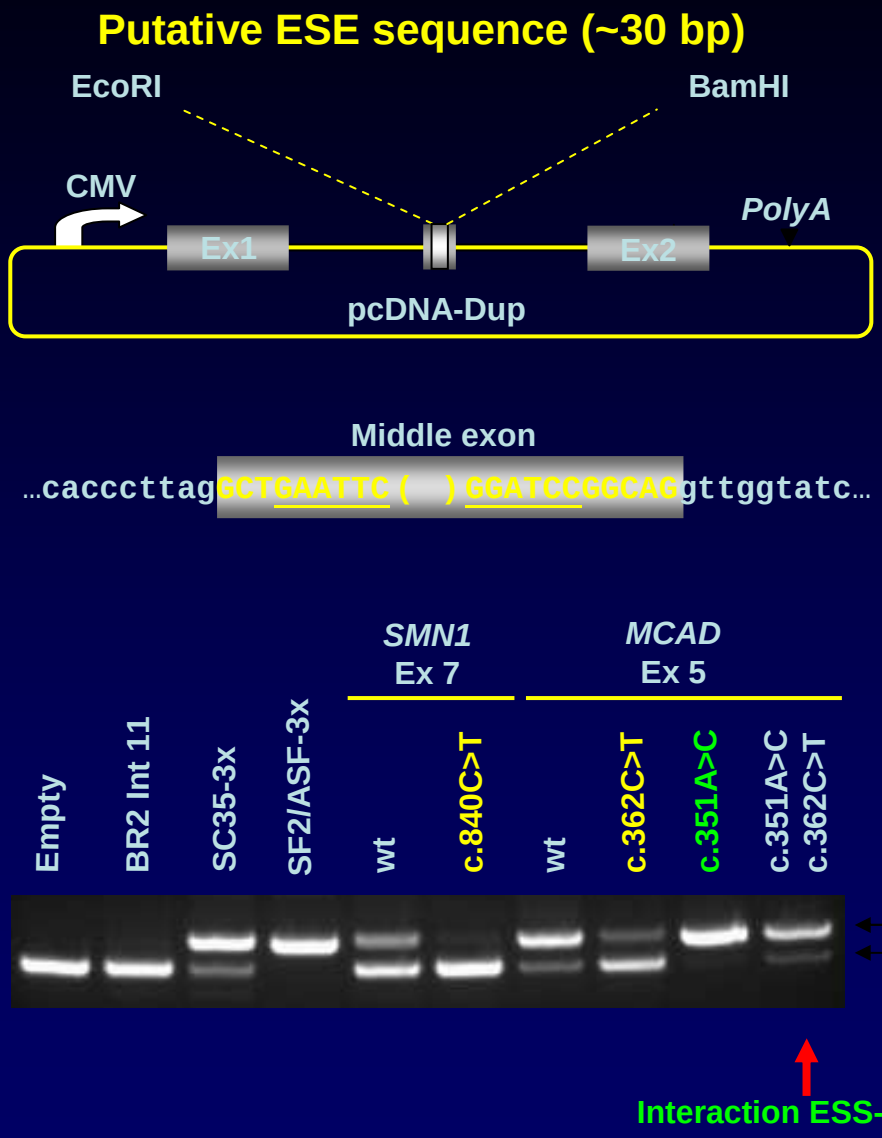
MUTATIONS OF PUTATIVE SPLICING REGULATORY ELEMENTS

Effect of four *MLH1* or *MSH2* exonic variants and one *MLH1* intronic UV in 3 different cell types



Same variants in Hela cells usig pSPL3 : similar results

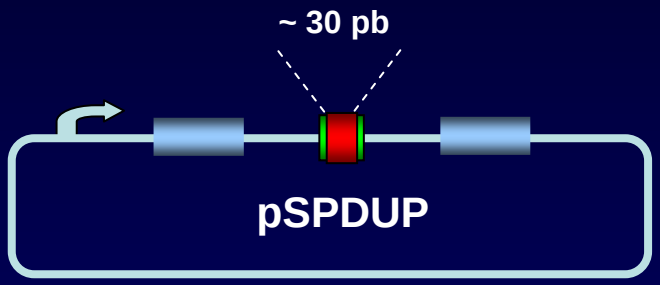
VALIDATION OF THE ESE-DEPENDENT SPLICING ASSAY



Nielsen et al. (Andresen BS) Am J Hum Genet 80:416 (2007)

IDENTIFYING ESE ELEMENTS OF *MLH1* AND *MSH2*

Sequences to be tested (~30bp) inserted into a central exon, which is strictly dependent on presence of a ESE



Wildtype sequence : exon inclusion

3 new ESE elements found in this work

Demonstration that 11 previously published mutations of *MLH1* ou *MSH2* are within ESE elements

Tournier et al. (2008). Human Mutation, in press

COMPARISONS WITH PATIENT RNA DATA

130 *MLH1*, *MSH2* variants analysed

25 % (31 / 130) variants affect splicing

37 / 130 variants tested on patient RNA

35 Concordant results

2 Discordant results

(but NMD not prevented)

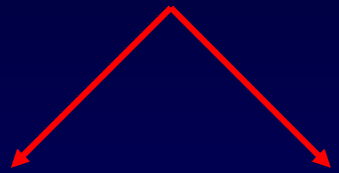
APPLICATIONS TO *BRCA1* AND *BRCA2*

French Network Breast cancer genetics

We compared patient RNA analyses and the minigene assay
Rouen + Caen (Dr. A. Hardouin)

20 variants *BRCA1, BRCA2* (17 families)

Analysed in parallel on



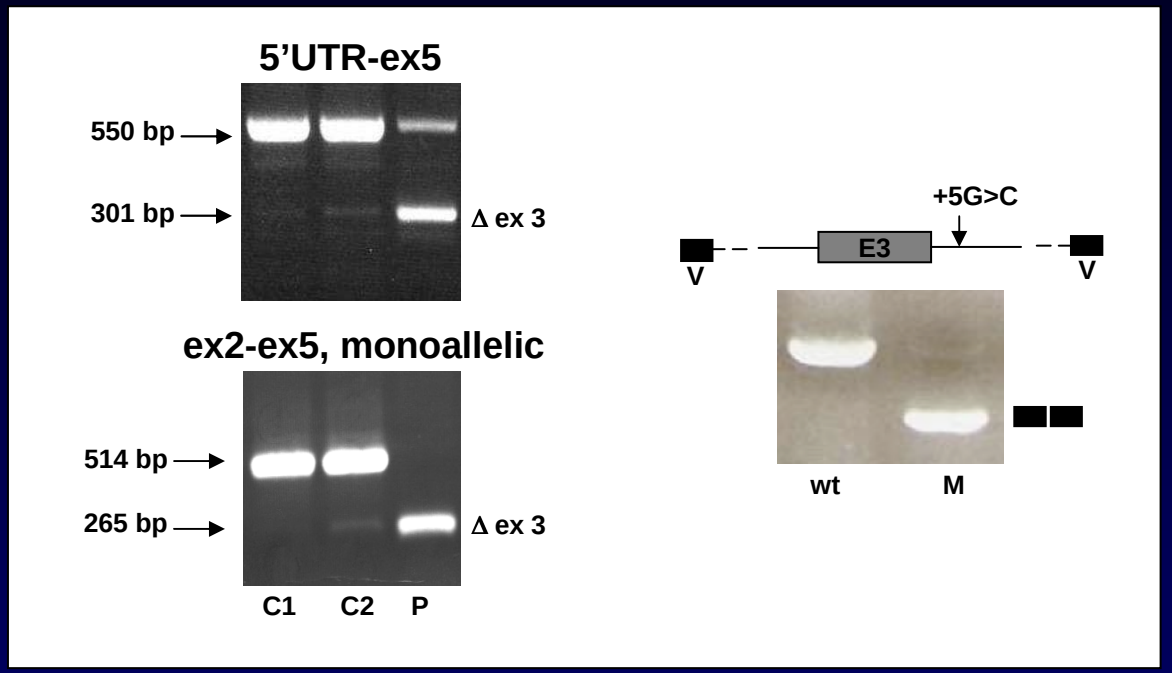
Patient RNA **Ex vivo minigene assay**

6 Variants affect splicing. Perfect agreement between *in vivo* and *ex vivo* data

EXAMPLE 1

BRCA2 c.316+5G>C, IVS3

Patient RNA **Minigene assay**



No normal mRNA expressed from mutant allele, **pathogenic**

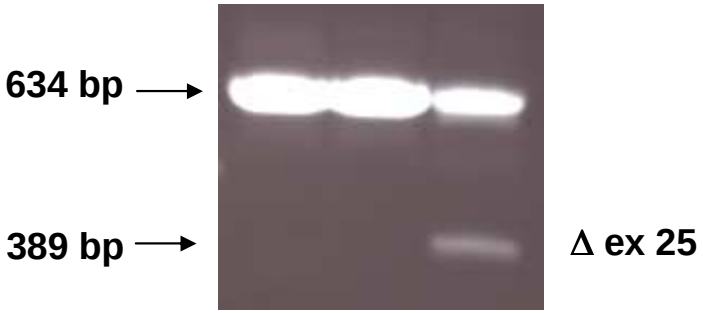
C. Bonnet, S. Krieger et al., revised manuscript submitted

EXAMPLE 2

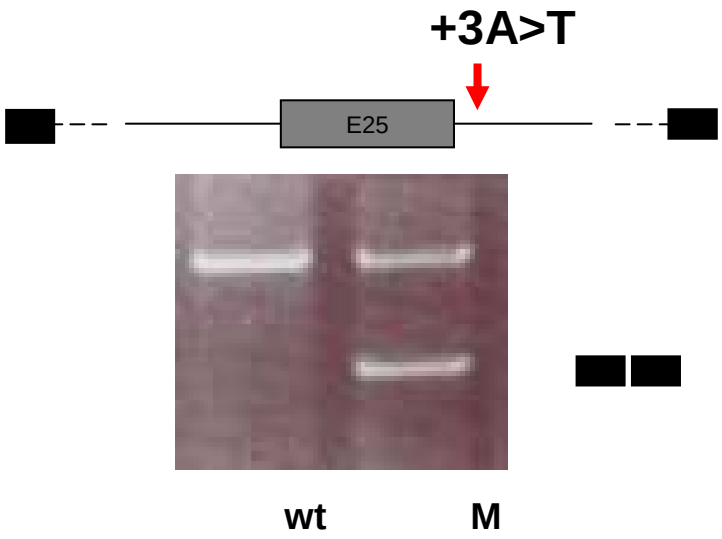
BRCA2 c.9501+3A>T (IVS25+3A>T)

Patient RNA

RT ex 23-ex 27



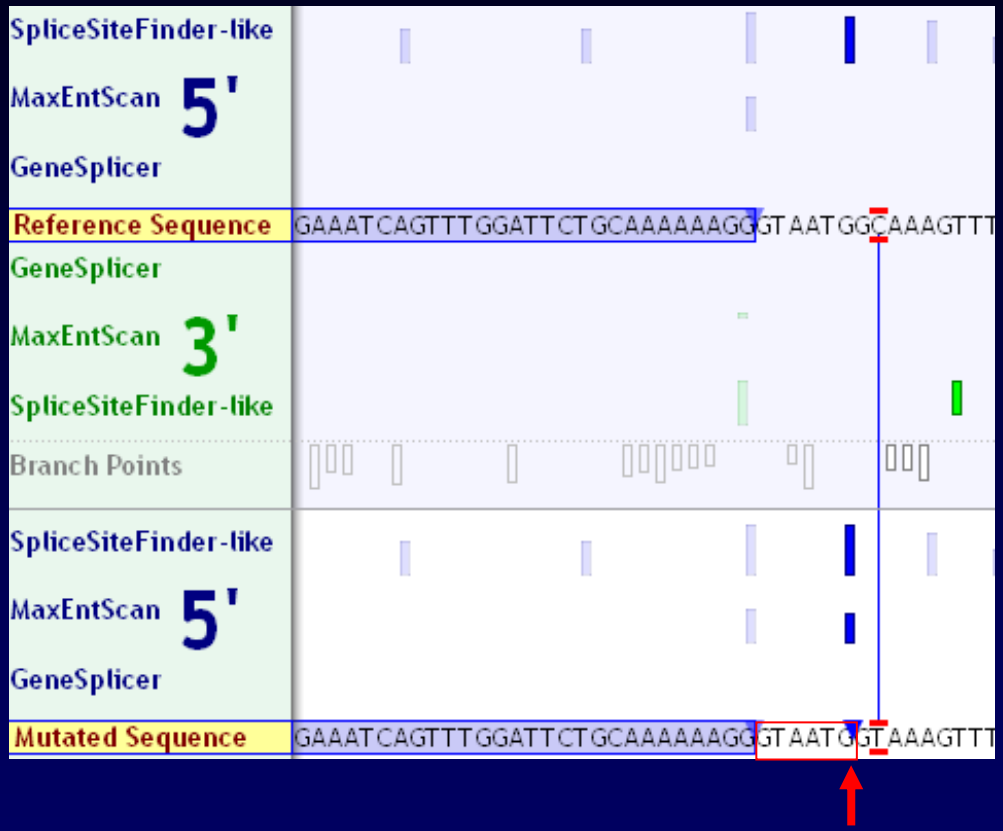
Minigene assay



Minigene assay : ~ 50% normal mRNA from mutant allele
Partial splicing defect, still unclassified

DISCREPANCIES BETWEEN *IN SILICO*, *IN VIVO* AND *EX VIVO*

BRCA1 c.670+8C>T (IVS10)



+ Automated Splice Site Analyses

Several in silico predictions : New Dss at +6, of same strength as natural one

RT-PCR on patient RNA : Only natural Dss used

pCAS Minigene splicing assay : Only natural Dss used

Unlikely to be pathogenic

But this variant promotes inclusion, both in vivo and in the minigene assay. Does it affect an ISS?

Using sofware « Alamut » from *Interactive Biosoftware*

SUMMARY

- **Large numbers of UVs screened with minigene assay**
  130 in *MLH1, MSH2* and 20 in BRCA1/2
- **Good concordance with patient RNA data**
  35/37 for *MLH1, MSH2* and 20/20 for *BRCA1/2*
- **25% of UVs classified as splicing defects**
  Mainly splice site alterations, activations of cryptic sites or generation of new sites
- **Five mutations found in new exonic regulatory elements**
- **Definition of 12 ESE-containing short regions**
  8 in *MLH1*, 4 in *MSH2*

ADVANTAGES OF *MINIGENE* ASSAY

- Very useful for **screening UVs**, because in most cases patient RNA is not available
- When patient RNA is available, minigene assay facilitates the interpretation, because **monoallelic**
- Although **exons are tested in heterologous context**, good concordance with patient RNA data
- Allows evaluation of ***in silico*** predictions
  - Algorithms predicting **5' and 3'** splice sites : **rather accurate**
  - Algorithms predicting **ESE, ESS** : neither sensitive nor specific

INTERPRETATION OFTEN REQUIRES

- **Adding genetic evidence:**
  segregation, co-occurrence of déléterious changes
- **Adding information obtained from tumours**
  (e.g. for HNPCC: IHC, microsatellite instability…)
- **Interrogating databases**
- **Frequent meetings of network members**

Inserm U614
Faculté de Médecine, Rouen
Service de Génétique, CHU Rouen

Isabelle Tournier
Myriam Vezain
Céline Bonnet
Audrey Killian
Alexandra Martins
Stéphanie Baert-Desurmont
Mario Tosi
Thierry Frébourg

Consultations d'Oncogénétique

Dominique Stoppa-Lyonnet
Institut Curie, Paris
Catherine Dugast
Centre Eugène Marquis, Rennes
RosetteLidereau,
Centre René Huguenin, FNCLCC, St-Cloud

Cancéropôle Nord-Ouest

Agnès Hardouin, Sophie Krieger
Centre François Baclesse, Caen

Réseau laboratoires HNPCC

Sylviane Olschwang
Institut Paoli-Calmettes, Marseille
Marie-Pierre Buisine
CHU, Lille
Qing Wang
Centre Léon Bérard, Lyon
Jean-Marc Rey
CHU Hôpital Arnaud de Villeneuve, Montpellier
Olivier Caron
CHU Hôpital Civil, Stasbourg
Christophe Philippe
CHU, Nancy
Stéphane Bézieau
CHU, Nantes

IGMM, UMR 5535, Montpellier

Johann Soret
Jamal Tazi