

# **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**

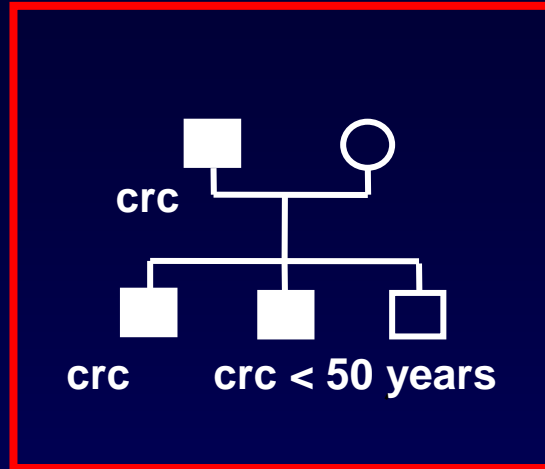
**London, 3rd March 2008**

**Eurasnet workshop : RNA splicing and genetic diagnosis**

**Workshop 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

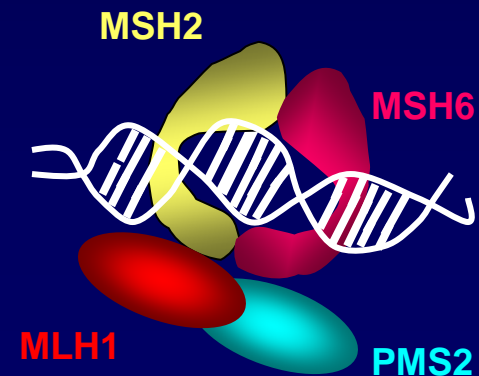
*MLH1* (3p21)

*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

<i>MLH1</i>	25 %
<i>MSH2</i>	25 %
<i>MSH6, PMS2</i>	5 %

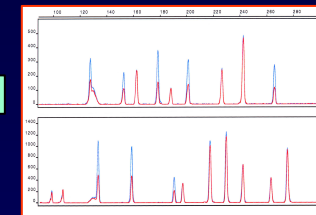
## - *MSH2* gene rearrangements

10 %

## - *MLH1* rearrangements

< 4 %

< 70 %



QMPSF

MLPA

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

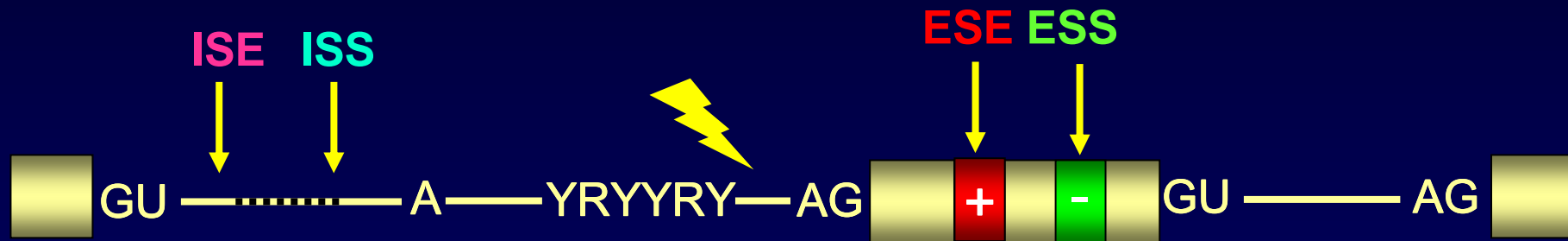
# SPLICING REGULATORY ELEMENTS

*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:**

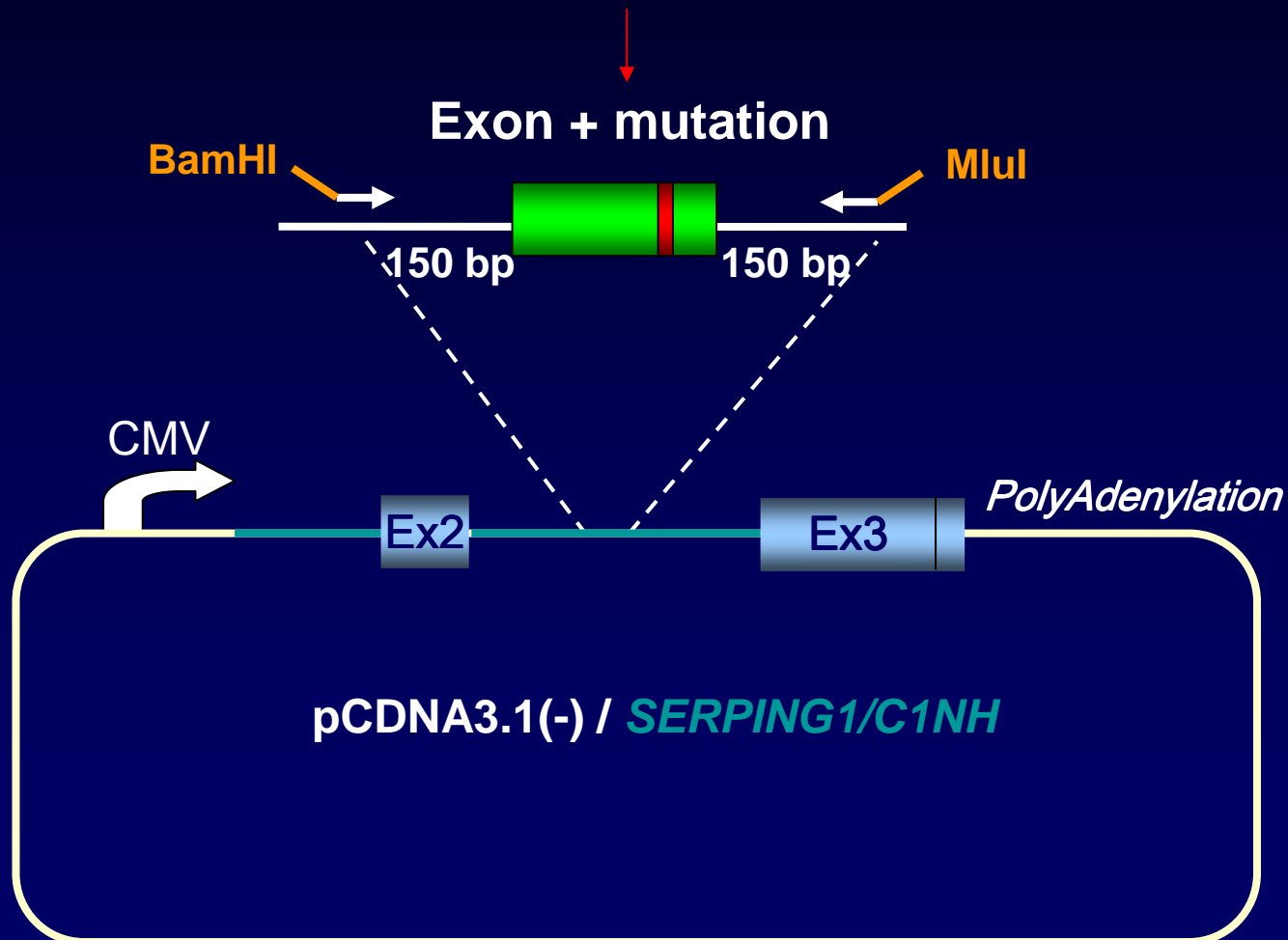
Baralle and Baralle *J. Med. Genet.* 2005;42;737-748

Cooper TA. *Methods* 2005; 37:331-340

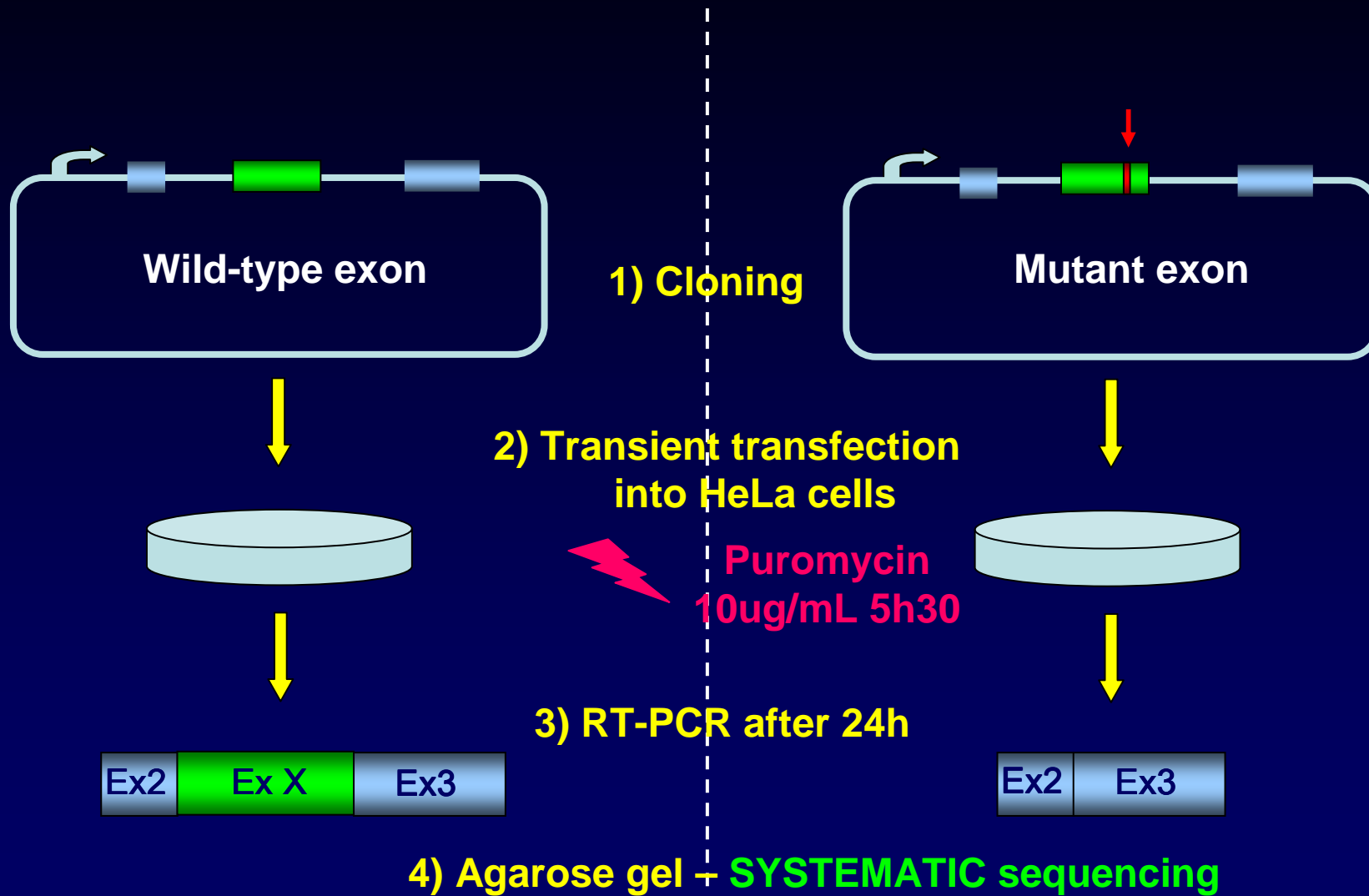
## SPLICING ASSAY

Patient blood samples (PaxGene) for RNA  
often not available

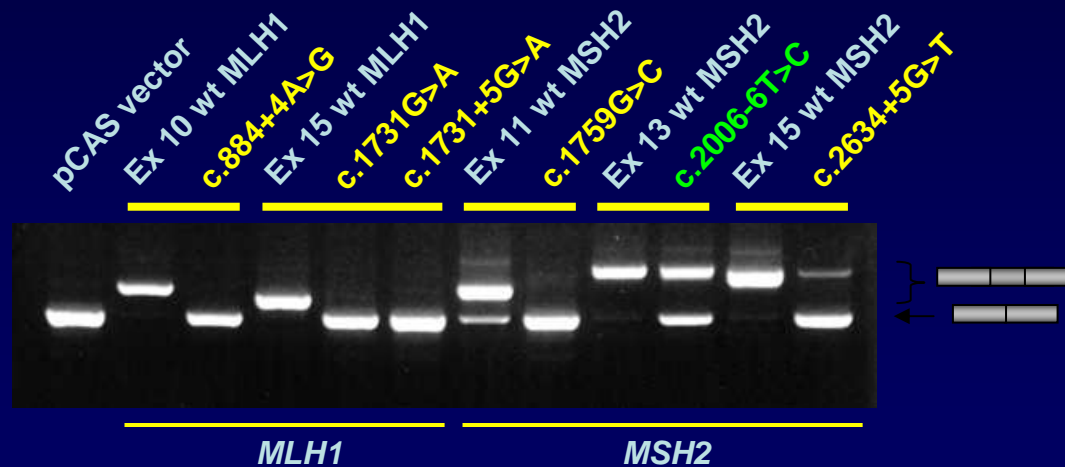
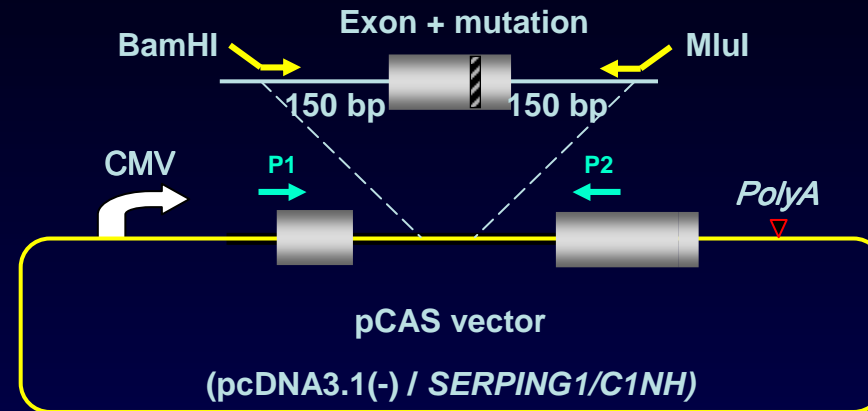
Patient DNA



# SPLICING ASSAY



# 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 cryptic splice site

**6** Mutations of putative splicing  
regulatory elements

# MUTATIONS OF PUTATIVE SPLICING REGULATORY ELEMENTS

*MLH1*

Exon 10, 3<sup>rd</sup> exon position  
4<sup>th</sup> exon position

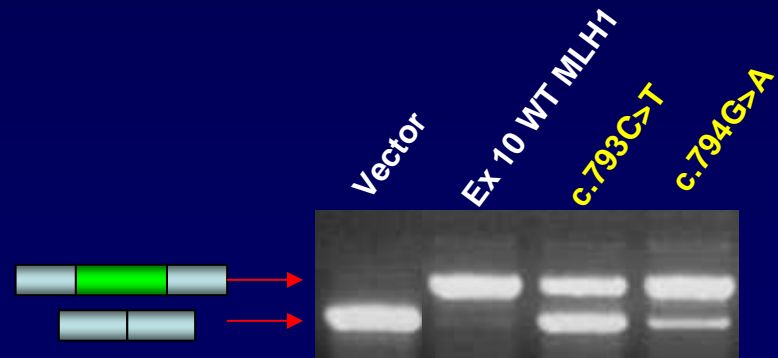
## ✦ In silico predictions

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 +++

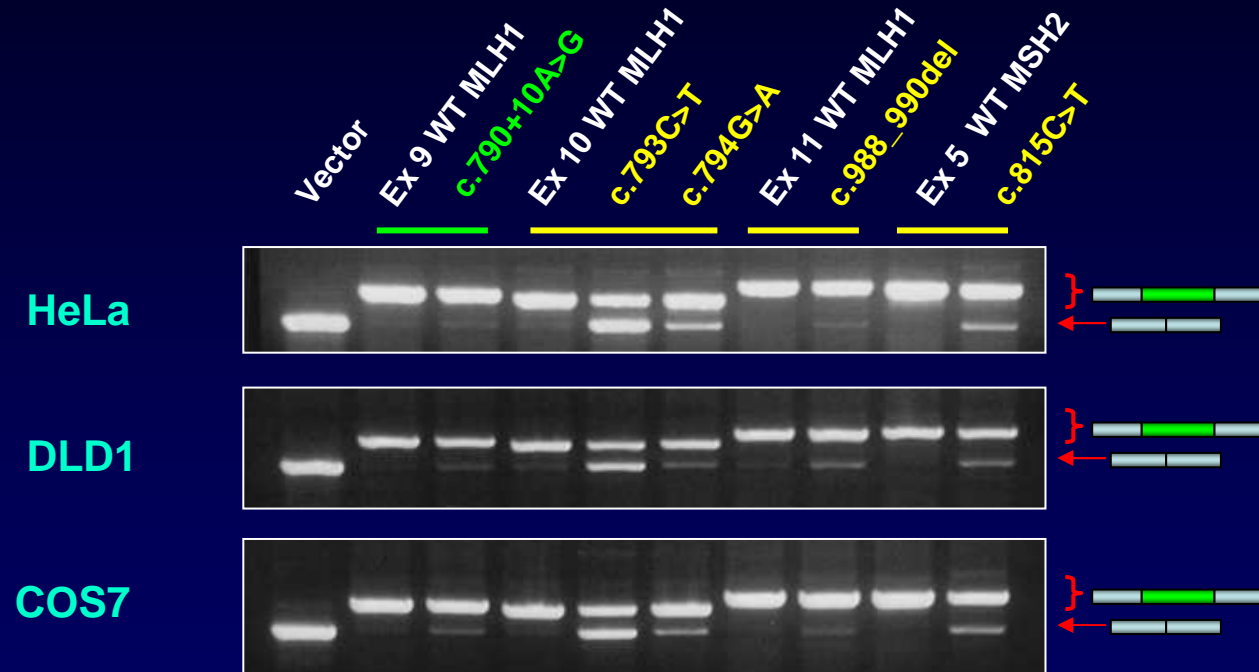
c.794G>A : partial exon 10 skipping ++



➡ Identification of a new ESE element ?

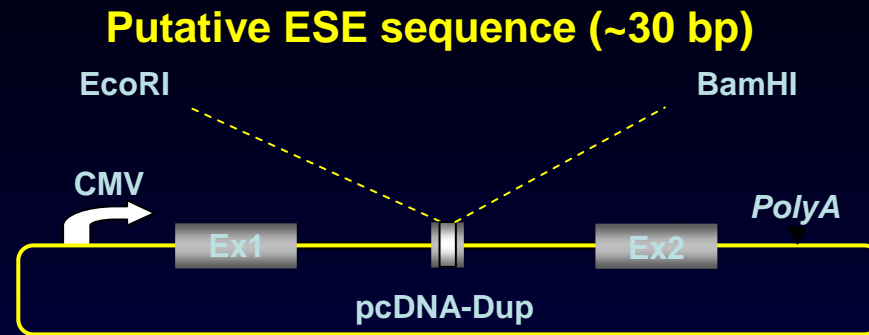
# MUTATIONS OF PUTATIVE SPLICING REGULATORY ELEMENTS

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



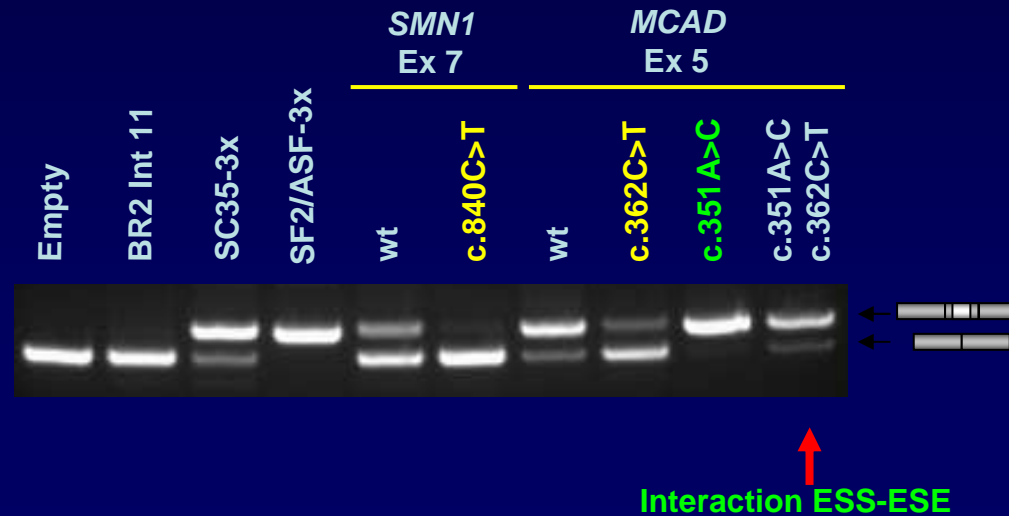
Same variants in HeLa cells using pSPL3 : similar results

# VALIDATION OF THE ESE-DEPENDENT SPLICING ASSAY



**Middle exon**

...cacccttag GCTGAATTC ( ) GGATCCGGGCAG gtttgtatc...

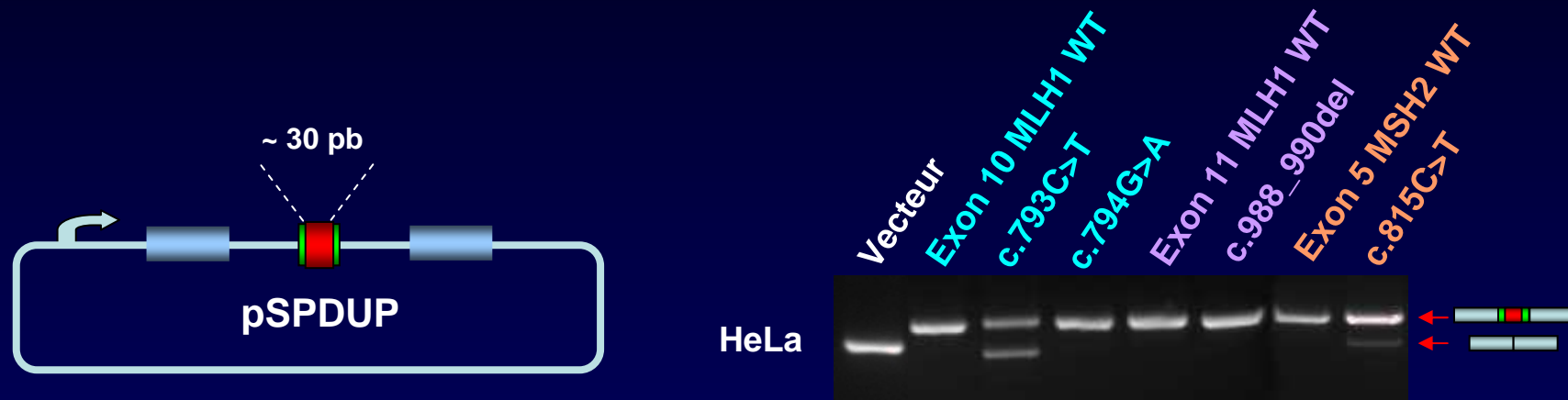


Nielsen et al. (Andresen BS) Am J Hum Genet 80:416 (2007)

## 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*

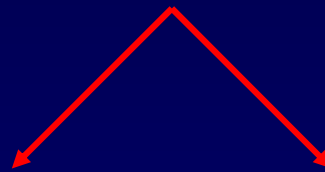
## COMPARISONS WITH PATIENT RNA DATA

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

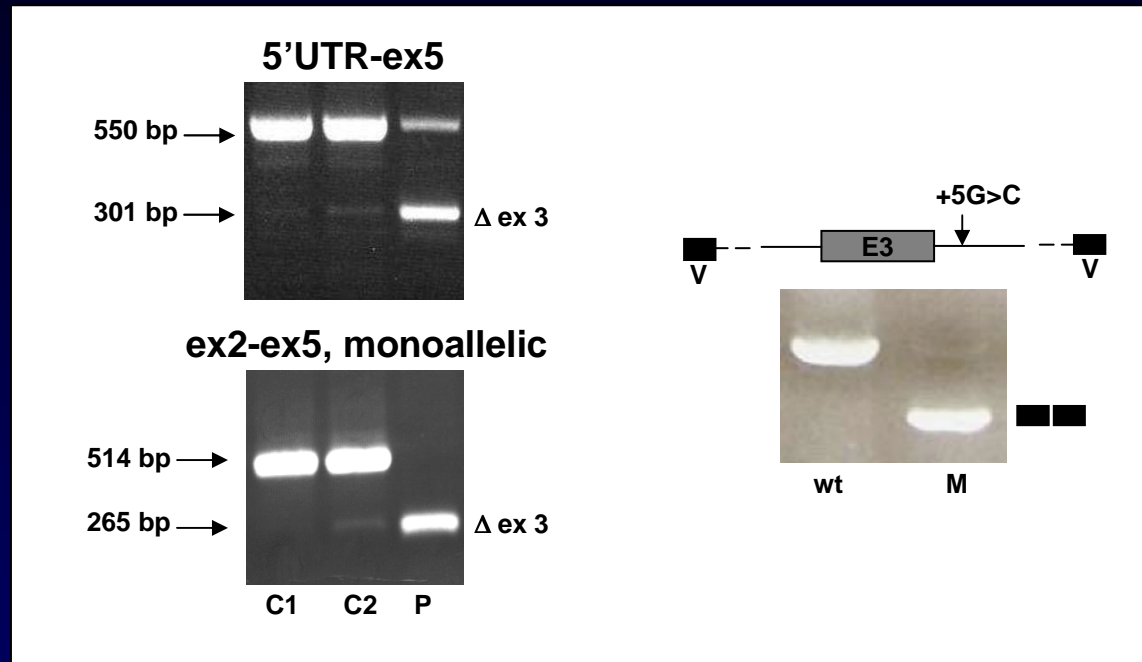
6 Variants affect splicing. Perfect agreement  
between *in vivo* and *ex vivo* data

# EXAMPLE 1

## BRCA2 c.316+5G>C, IVS3

Patient RNA

Minigene assay

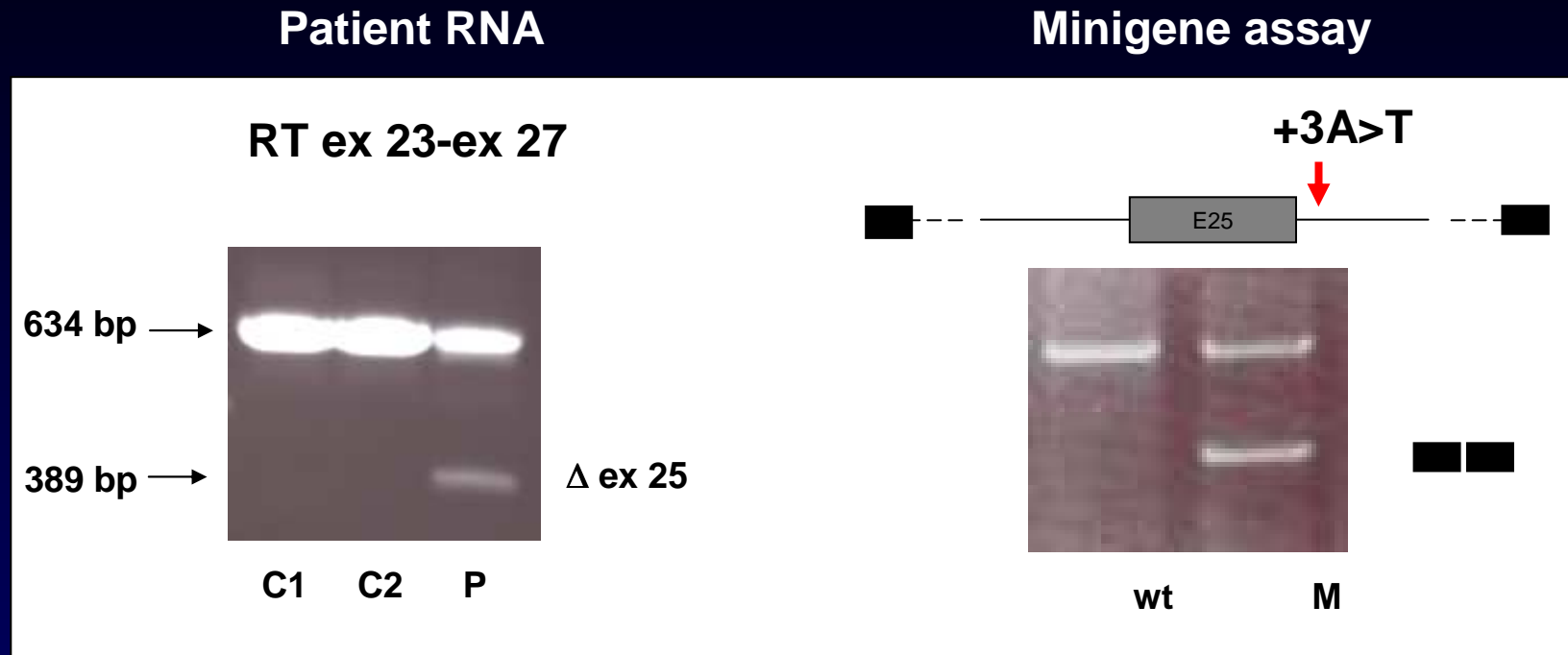


No normal mRNA expressed from mutant allele, pathogenic

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

## EXAMPLE 2

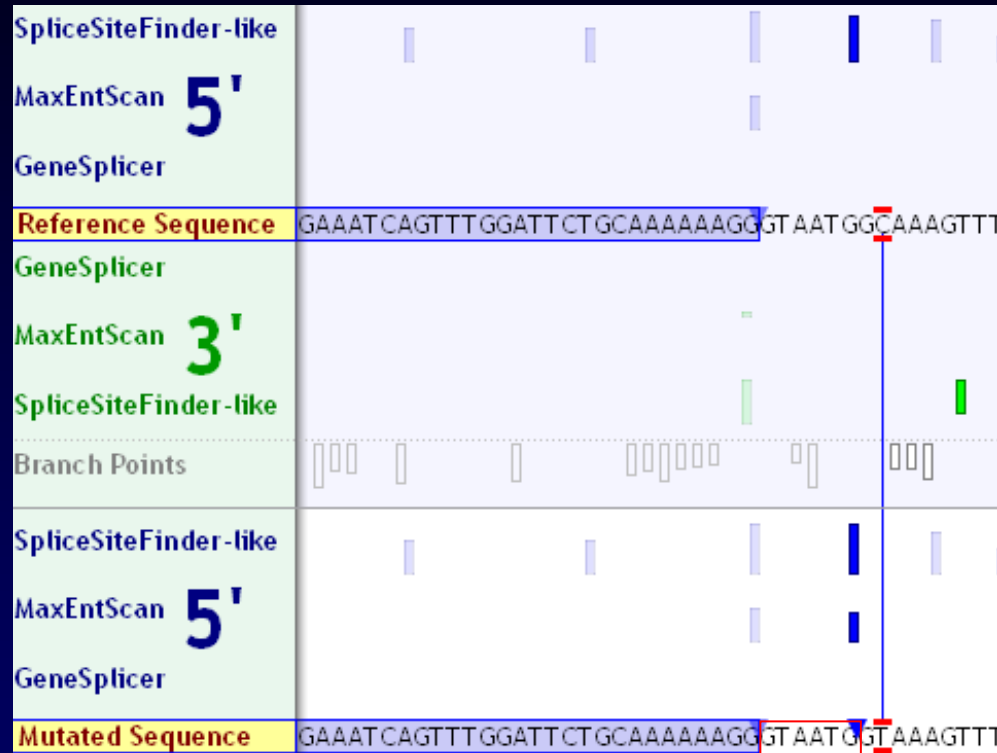
### *BRCA2* c.9501+3A>T (IVS25+3A>T)



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 software « 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 délétérious 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  
**Rosette Lidereau,**  
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**