Original article

Pathogenic intronic and deleterious benign variants: two extremes in cancer predisposition molecular diagnosis

Variantele intronice patogenice și deletere benigne: două extreme în diagnosticul molecular al predispoziției la cancer

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Abstract

Molecular diagnosis in cancer predisposition is today current practice in Western Europe, which allows oncogenetic follow-up of patients and their families. Diagnosis is mainly targeting BRCA, MMR and APC genes, involved in hereditary breast and ovarian cancer syndrome (HBOC), hereditary non-polyposic colorectal cancer or Lynch syndrome (HNPCC), and familial adenomatous polyposis (FAP) respectively. Carriers of deleterious mutations in any of these genes are at significantly higher risk of developing cancer than general population. Thousands of BRCA sequence variations have already been reported, but not all variants can be considered pathological. Deleterious mutations and common non-pathogenic single nucleotide polymorphisms are usually detected, but almost a half of the observed variations are of uncertain clinical significance. In-silico analysis including sequence alignments, tolerance prediction and splicing analysis is therefore essential for understanding possible effects on protein function and pathogenicity. While completely sequencing BRCA1 and BRCA2 genes in routine molecular diagnosis, we found several unclassified variants which easily can blur analysis, either being false-positive or false-negative. We show here that extreme examples such as pathogenic intronic and deleterious benign variants represent real challenges in molecular diagnosis. Good experience, a lot of attention and responsibility are essential in order to avoid errors.

Keywords: molecular diagnosis, unclassified variants, in-silico analysis, false positives, false negatives.

Rezumat

Diagnosticul molecular al predispoziției ereditare la cancer este la ora actuală o practică standardizată în lumea occidentală, care permite monitorizarea oncogenetică a pacienților și a familiilor acestora. Diagnosti-

***Corresponding author** : Lucian Negura, University of Medicine and Pharmacy Grigore T Popa of Iasi, Immunology department,Str. Universității, nr.16; 700115 Iasi Tel. +40 747 502728, E-mail: luciannegura@yahoo.fr cul vizează cu precădere genele BRCA, MMR și APC, implicate respectiv în sindromul predispoziției ereditare la cancerul colorectal nonpolipozic (HNPCC), și polipoza adenomatoasa familială (PAF). Purtători ai unor mutații deletere la nivelul oricărei gene de mai sus prezintă un risc semnificativ mai crescut de a dezvolta un cancer, comparativ cu populația generală. Mii de variante de secvență BRCA au fost raportate în bazele de date până la ora actuală, însă nu toate pot fi considerate patogenice. În mod obișnuit sunt detectate mutații deletere și polimorfisme comune nepatogenice, însă aproape jumătate dintre variantele de secvență detectate prezintă o semnificație clinică incertă. Din acest motiv, analiza in-silico, incluzând alinieri de secvență, predicția toleranței sau evaluarea splicingului, este esențială pentru înțelegerea posibilelor efecte asupra funcției proteice și asupra patogenicității. Prin secvențierea completă a genelor BRCA1 și BRCA2 în cadrul diagnosticului molecular de rutină, am identificat mai multe variante de secvență neclasificate, ce ar putea ușor contribui la un diagnostic eronat, fie prin fals-pozitivitate, fie prin fals-negativitate. Prezentăm în acest material exemple extreme precum variantele intronice patogenice și deletere benigne, adevărate provocări în diagnosticul molecular. O bună experiență, multă atenție și responsabilitate reprezintă atuurile în evitarea erorilor de diagnostic.

Cuvinte cheie: diagnostic molecular, variante neclasificate, analiză in-silico, fals positive, fals negative. *Received:* 21st November 2012; *Accepted:* 3rd December 2012; *Published:* 15^h December 2012.

Introduction

Molecular diagnosis of cancer susceptibility genes is nowadays widely applied in clinical practice to evaluate hereditary risk factor of developing cancer (1). Usually, the diagnosis is based on DNA sequencing, in order to identify genetic variation possibly involved in the alteration of normal protein functions. Molecular testing is currently available for hundreds of genes, or genetic conditions involved in more or less common cancer syndromes. Cancer predisposition diagnosis is mainly targeting BRCA, MMR and APC genes, involved in hereditary breast and ovarian cancer syndrome (HBOC), hereditary non-polyposic colorectal cancer or Lynch syndrome (HN-PCC), and familial adenomatous polyposis (FAP) respectively (2-4). Carriers of deleterious mutations in any of these genes are at significantly higher risk of developing cancer than general population (5-7). Therefore, molecular diagnosis in cancer predisposition is today current practice in Western Europe, which allows oncogenetic follow-up of patients and their families (8).

Although DNA sequencing by chain-termination method was developed by Frederick Sanger in 1977, and even if his method quickly became a "gold-standard" for the lecture of DNA primary structure, its use was limited for many years to research activities, and was not very used for clinical purposes such as genetic tests. High expenses, lack of reference sequence information and difficulties in interpreting sequence data were the principal inconvenient for the link between sequencing of the entire coding region and clinical molecular diagnosis (9). The situation extraordinarily evolved in the last decades, and due to technological advances, sequencing of whole human genome, and serious diminution of costs, DNA sequencing became widely used, especially in investigating the coding region of tumor suppressor genes (10).

In Hereditary Breast and Ovarian Cancer (HBOC), germ-line mutations predisposing to the disease are mainly affecting BRCA1 (OMIM 113705) and BRCA2 (OMIM 600185) genes as principal responsible in over 1/3 of hereditary cases, that mean familial agglomerations with 2 or more early onset breast or/and ovarian cancers (11). Full sequence analysis of the BRCA genes, available since 1996, was one of the first sequence-based tests offered to evaluate hereditary risk for common forms of cancer (12). It is nowadays standard diagnosis for HBOC families, underlying whole oncogenetic consulting and follow-up. The two genes are very large, composed of thousands of coding nucleotides sparing 100 kb genomic DNA each. As an additional difficulty, both genes possess

an unusually large exon 11, of 3,4 kb in *BRCA1* and 5 kb in *BRCA2*. Even though limiting sequencing to exonic regions and exon/intron boundaries, there is a huge amount of work to do when attempting to completely investigate *BRCA1* and *BRCA2*. That will comprise a total of 84 amplicons to be forward and reverse sequenced, which means a very expensive and time consuming approach (13-15).

Unfortunately, more than a half of thousands sequence variants reported in common databases (BIC (16); UMD (17)) are of uncertain clinical significance (UVs), which are generally single nucleotide silent or mis-sense substitutions, in-frame modifications, or intronic variations. This is a situation "not-so-easy" to manage in oncogenetic management (18-20). Several approaches have been proposed to resolve unclassified variants, including *in-silico* prediction, segregation analysis or functional tests (18-20).

While completely sequencing *BRCA*1 and *BRCA*2 genes in routine molecular diagnosis (13-15), we found several unclassified variants which easily can blur analysis, either being false-positive or false-negative. We show here that extreme examples such as pathogenic intronic and deleterious benign variants represent real challenges in molecular diagnosis. Good experience, a lot of attention and responsibility are essential in order to avoid errors.

Patients and methods

Patients

We identified and recruited predisposition HBOC patients at the Sf. Spiridon University Emergency Hospital of Iaşi, Romania, as well as at the Oncolgy Institute of Cluj-Napoca, Romania. We used for this study results data from over 50 breast cancer cases, including familial, early-onset (< 40 years), male breast cancer, and bilateral cases.

For the characterization of HBOC cases, we used criterions previously described (13), i.e. three or more breast or ovarian cancer cases within the same family branch. We also

considered for analysis early onset cancer cases (breast – before 40; ovarian – before 60), as well as multiple (including bilateral) or male breast cancer cases. All patients agreed by written informed consent. Personal and familial cancer histories were obtained from patients and participating relatives.

Molecular analysis

We performed genomic DNA extraction as previously described (13), using the WizardTM Genomic DNA purification kit (PromegaTM Inc, Madison, WI, USA). Spectrophotometric evaluation of DNA quantity and purity was used.

Sanger dideoxy sequencing was performed on 84 amplicons covering the whole coding sequence of BRCA1 and BRCA2, including junctions with introns. DNA sequencing was performed in collaboration within the Molecular Epidemiology Laboratory, University of Medicine and Pharmacy Gr. T. Popa Iasi, Romania, and the Molecular Oncology Laboratory, Centre Jean Perrin, Clermont-Ferrand, France. PCR was performed in 20 µl reaction, containing one unit ApliTaq® Polymerase with appropriate Buffer (Applied Biosystems[™] Inc, Foster City, CA, USA), 0.4 mM each dNTP, 0.8 µM of each primer, 75 ng genomic DNA. We generally performed PCR reaction on an EppendorfTM Mastercycler®, 94°C/5min, 30 cycles of 94°C/20 sec -54°C/20 – 72°C/30 sec, 7 min/72°C.

After gel electrophoresis evaluation, amplicons were purified by Ampure[®] reagents on Biomek[®] FXP workstation (Beckman CoulterTM Inc, Brea, Ca, USA), following producer's instructions. Amplicons ware sequenced both in forward and reverse reactions, using the BigDye[®] Terminator Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's instructions. Sequencing reaction was performed on a 96-Well GeneAmp[®] PCR System 9700 (Applied Biosystems), 94°C/11 min, 25 cycles of 94°C/10 sec – 52°C/5sec - 70°C/3min. Sequence products were purified using automatized CleanSeq[®] system on Biomek[®] FXP workstation (Beckman Coulter), and migrated by capillary electrophoresis on an



Figure 1c. BRCA1 exon 7 (underlined) in the amplicon context

ABI 3130XL DNA analyser (Applied Biosystems). We performed analysis of raw data using Seqman[®] (DNA Star[™] Inc, Madison, WI, USA). Mutations were systematically confirmed on an independent different DNA sample.

In-silico analysis

All mutations and sequence variants are described according to the recommendations from the Human Genome Variation Society (HGVS), with first nucleotide of DNA numbering being the A from initiator translated ATG (21). We used reference sequences NM_007294.3 for *BRCA1* and NM_000059.3 for *BRCA2*. For international database consulting, we used either the BIC (Breast Information Core) Database (16) or the UMD (Universal Mutation Database) (17).

For bioinformatic prediction of variants, we used Alamut[®] (Interactive SoftwareTM) (22). This software includes GVGD[®] Alignment (*Grantham Variation – Grantham Deviation*), SIFT[®] (*Sorting Intolerant From Tolerant*) or PolyPhen[®] (*Polymorphism Phenotyping*). Splicing simulations were performed using SpliceSiteFinder[®] (SSF), MaxEntScan[®], NNSplice[®], and GeneSplicer[®], as well as ESE finder for Exonic Splicing Enhancer sites.

Results

We present, by some concrete examples, the importance of thorough interpretation of sequence variants. Both false positives (cases no. 1



Figure 2b. Prediction on the protein effect of the observed BRCA2 delins variation

and 2) and false negatives (cases no. 3 to 5) are presented, in order to offer a more complete, although non-exhaustive, image of interpretation difficulties. A special attention will be given to cases no 2 (called deleterious benign variant) and 5 (called pathogenic intronic variant), as considered extreme situation of molecular diagnosis.

1. Case no. 1 – The "technical" false positive

Heterozygous frameshifts usually appear as "dirty" or doubled sequences, while a "clean" and simple capillary electrophoretic profile is indicating the wild-type homozygous sequence. Therefore, the interpreting biologist will be more often tempted to attribute a frameshift value to any doubled sequence, as the one which can be observed in *Figure 1a*, corresponding to the reverse dye terminator sequencing of *BRCA*1 exon 7.

However, the supposed deleterious variation proves to be present in more than one patient (actually in all samples), which could be, in a second stage, easily be attributable to contamination. The forward sequencing of the same amplicon shows no frameshift at the same position (*Figure 1b*), but a different "dirty" sequence 3' downstream, this again in all samples. What could be the cause?

A closer look to the sequence of the amplified exon 7 shows a T and C repetitive region of about 40 nucleotides (*Figure 1c*). In fact, this causes a slippage of the Taq polymerase, either generating a whole doubled sequence in reverse sequencing, or a terminal doubled sequence in forward sequencing. Since the coding region of interest (*Figure 1c*, underlined) is at a fair distance of the repeated region, exon 7 can still be sequenced by using a high fidelity polymerase, but only in forward sequencing. This could easily trouble interpretation when a real frameshift is affecting exon 7.

Primers are shown by black arrows.

2. Case no. 2 – The real false positive

As shown above, a doubled sequence usually indicates a frameshift heterozygous variation, as is the case on can observe for BRCA2 exon 27, in Figure 2a. By in-silico analysis and Alamut interpretation, the variation really showed to be deleterious, affecting the sequence simultaneously by one nucleotide deletion and 11 nucleotide insertion. This was called c.10095delinsGAATTATATCT or c.10095del-Cins11. At the protein level (Figure 2b), the variant causes frameshift starting with a Lys to Asn substitution in position 3366, and a premature termination at position 3370, which truncates the BRCA2 protein of its C-terminal 49 aminoacids.

The most interesting aspect of our example is given by the proven neutrality of the c.10095del-Cins11 variant, otherway a clear deleterious mutation. This can only be deducted by database analysis. As a matter of fact, another *BRCA2* terminal deleterious mutation, the c.9976A>T (p.Lys3326STOP) variant was proven to be clearly non pathogenic, by



Figure 3. Visibility of a SNP in forward sequencing (up) and reverse sequencing (down)

several studies reported in the UMD database (17). A possible explication is that *BRCA2* protein may lack some C-terminal aminoacids and still keep its entire biological functions. It is logical to deduce that any deleterious variants found 3' downstream c.9976A>T (which is the case for our c.10095del-Cins11) will either be non pathogenic. This has been also proven by some other reports of c.10095del-Cins11 with no clinical significance, as presented in the BIC database (16). We called our variant a "deleterious benign".

The non-pathogenicity of a deleterious mutation is an outstanding example on the importance of thorough interpretation either by *insilico* tools and database consulting.

3. Case no. 3 – The visual false negative

The vast majority of variants observed in BRCA genes are single nucleotide substitutions. Although a lot are just polymorphic nonpathogenic variants, sometimes a single SNP can have deleterious effect by creating a stop codon, or by affecting an essential aminoacid of the protein. On the other hand, heterozygous SNPs are the most difficult to observe in electropherograms, as doubled peaks may sometimes not be observable, if perfect superposition of the two sequences occurs. We show in figure 3 such an example of a G (black peak) for A (green peak) substitution, which shows the importance of double sequencing forward and reverse (the SNP is easily observable in reverse sequence while almost invisible in forward sequencing).

4. Case no. 4 – The artefact false negative

Figure 4 presents an example of artefacts which may interfere with a correct interpretation, especially in cases where electropherogram peaks are not very distinct. In such situations, pre-peaks appearing from highly detected colours superpose with real peaks from low detected colours, generating a false double-peak which can be found in the whole sequence. This can be a real problem when a SNP or mutation within the same sequence is affecting nucleotides detected by the same colours as the pre-peaks. Generally, a confirmation by reverse or forward sequence is needed and can clarify the situation.

5. Case no. 5 – The real false negative

The whole introme sequencing is barely imaginable for *BRCA* genes, while it should generate hundreds of intronic variants, either homozygous or heterozygous. Therefore, the attention in sequence interpretation is principally focused on exome analysis. Intronic variants are usually ignored or misunderstood. We show in *Figure 5a* an intronic substitution affecting the 5' upstream *BRCA2* exon 13.

The c.6938-1G>A substitution is novel and no reports exist in UMD or BIC databases, Therefore, a first Alamut splicing analysis was performed (*Figure 5b*). As we generally take in consideration the MaxEntScan evaluation, we firstly observed a diminution of the splicing acceptor site force, from 5.5 to 2.2, which generally is not a quite important score to be considered (a strong splicing site has a MaxEnt score above 7). How-



Figure 4. SNPs can be mistaken for pre-peaks when affecting the same nucleotides

ever, an on-line MaxEnt application called "Max-EntScan::score3ss" simulated a slightly different effect, by evaluating a modification of the splicing acceptor score from 5.5 to -3.2 (*Figure 5c*).

By taking a more attentive look at figure 5b, one can observe that the 3' acceptor site before the substitution (up) is not at all the same as the splicing site after the substitution (down). In fact, a AG dinucleotide is always necessary for acceptor sites. When substituting the G with an A, a novel AG appeared, whith the G nucleotide being exonic ! Therefore, the old splicing site is not decreasing in force, but is replaced by a novel site (Figure 5d). Moreover, since the first exonic G nucleotide belongs to the novel site, it disappears from the exon, and a clear frameshift will affect the entire coding region downstream. This proves that a simple intronic substitution can be in fact a massive deleterious mutation by frameshift. We called it a "deleterious intronic", and we belive it represents another outstanding example on the importance of thorough interpretation either by in-silico tools.

Discussion

When completely sequencing and interpreting the coding region of a gene, three possible results can be obtained and communicated to phys-

icians or patients: (a) deleterious mutations, i.e. positive testing - the protein function is altered, and clinical consequences are very likely to be involved; (b) common polymorphisms or no sequence variation, i.e. negative testing - no consequence on protein structure and function; (c) uncertain - "variant of uncertain significance or unclassified variant" (UV), when there is no clear answer about pathogenicity and clinical implication of the variant (23). There is a real challenge in interpreting, classifying and communicating unclassified variants to the clinicians (and implicitly to patients) (24, 25). The responsibility of laboratory diagnostic is all the more so important, as clear answers are required, while classification systems upgraded to 5-levels, from 5 (definitely pathogenic) to 1 (not pathogenic at all), as shown in *Table 1* (9).

In routine molecular diagnosis, errors can appear at different levels, possibly affecting interpretation or even disturbing the whole analysis. Common manipulation errors may affect all steps from DNA extraction, through PCR amplification and purification, DNA sequencing and purification, to data transfer and interpretation. Interpretation errors point mainly as false positives (detection of inexisting mutations, or overrating of UVs) or false negatives (undetection of pathogenic mutations, or undervaluing of UVs).



Figure 5a. Detection of an intronic c.6938-1G>A within BRCA2 intron 12



Figure 5b. Evaluation of BRCA2 c.6938-1G>A by Alamut splicing simulation

ATAAAATAATTGTTTCCTAGGCA	MAXENT :	5.54	MM :	5.64	WMM:	2.78
ATAAAATAATTGTTTCCTA <mark>A</mark> GCA	MAXENT :	-3.21	MM:	-3.11	WMM:	-5.97

Figure 5c. Evaluation of BRCA2 c.6938-1G>A by MaxEntScan::score3ss

Acceptor Sites							
	SSF [0-100]	MaxEnt [0-16]	NNSPLICE [0-1]	GeneSplicer [0-15]	HSF [0-100]		
Threshold	> 70	> 0	>0.4	20	× 60		
Intron 12 - c.6938-59	86 88	6.46	0.84		84.46		
Intron 12 - c.5939-39	- 72 29	= 4.43	- 0.57		- 79 82		
Exon 13 c.6938 N	83.29 ⇒ —	5.64 ⇒ —			82.45 ⇒ —		
Ехол 10 - с.6909	⇒ 71.70	— ⇒ 2.21					
Fano 13 - 6 0050					- 68.15		
Ezon 13 - 6.0957					- 66 52		
Excn 10 - c.6900	- 82.91	- 6.94	- 0.68		- 80 47		
Excn 10 - c.6905	- 00.36	- 0.33			- 79.91		
Introv 13 - c.7997-6			<u> </u>		= 75.15		

a atural Splice Site

Figure 5d. Thorough evaluation of BRCA2 c.6938-1G>A by Alamut splicing simulation

Class	Description	Probability of being Pathogenic
5	Definitely Pathogenic	>0.99
4	Likely Pathogenic	0.95–0.99
3	Uncertain	0.05-0.949
2	Likely Not Pathogenic or of Little Clinical Significance	0.001-0.049
1	Not Pathogenic or of No Clinical Significance	< 0.001

Table 1. Classification System for Sequence Variants Identified by Genetic Testing (13)

False positive often occur by technical reasons or by insufficiently clear sequence detection. Still, we presented an uncommon non-pathogenic deleterious mutation, which can only be interpreted by referring to international databases. At the other extreme, false negatives occur generally by user's fault or lack of attention. We especially noticed a apparently minor intronic substitution, with devastating effect on protein structure.

Between deleterious benign and pathogenic intronic, the world of sequence variants is every day a source of difficulties and thorough analysis. Interpretation of sequence data is not quite a nice game (although it looks like), excellent knowledge of molecular principles of life being essential for a molecular reasoning.

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