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High-throughput characterization of *BRCA1* and *BRCA2* splicing defects

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BACKGROUND

Advances in DNA Next Generation Sequencing (NGS) techniques resulted in the detection of many thousands of germline unclassified variants with unknown functional impact (1). Variants of unknown significance (VUS), especially in clinically actionable genes such as the hereditary breast and ovarian cancer susceptibility genes *BRCA1* and *BRCA2* (OMIM 113705 and 600185 respectively), pose significant challenges to the medical community and patients (2). Among the variants that are frequently classified as VUS are those with unclear effects on splicing (3). Currently, there are no cost effective and high-throughput assays to quantify and characterize germline splicing defects in a time-frame necessary for clinical testing. Here we discuss the validation and implementation of a novel high-throughput RNA-NGS assay designed to perform *quantitative* and *qualitative* characterization of splicing VUS.

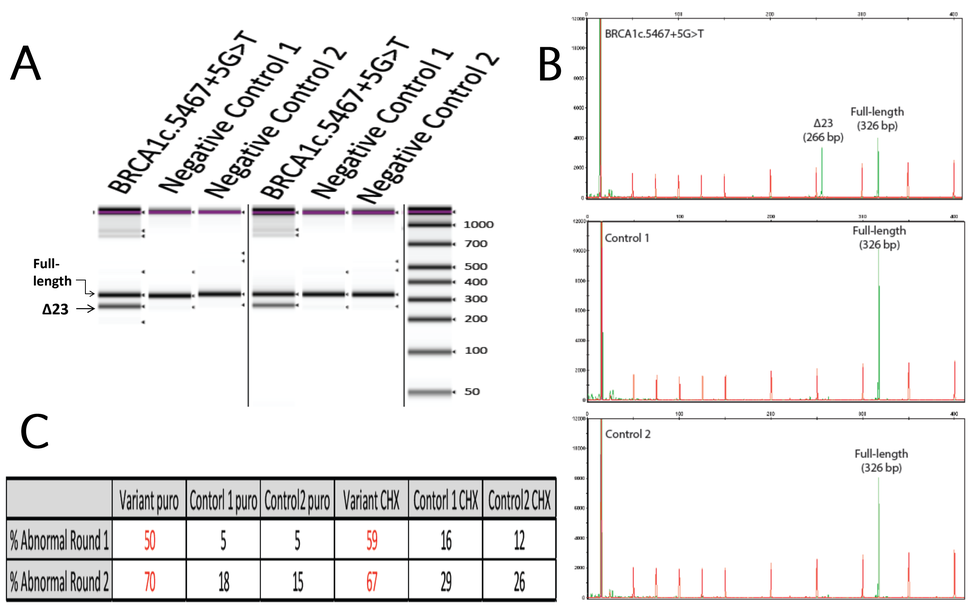
METHODS

We compared the gold-standard RNA splicing assays recommended by members of the ENIGMA (Evidence-based Network for the Interpretation of Germline Mutant Alleles) consortium (4), including Capillary Electrophoresis (CE) and Sanger sequencing of sub-cloned transcripts, to high-throughput RNA-NGS assays. Following the ENIGMA protocol we performed RT-PCR cDNA analysis of lymphoblastoid cell lines (LCLs) generated by the kConFab consortium from carriers of *BRCA1* or *BRCA2* variants known to be associated with splicing defects, of control LCLs, and patient blood samples. In parallel, we performed RT-PCR RNA-Seq. RNA-Seq reads were mapped to the genome reference (hg19) using STAR (version 2.5.2a) and processed by our custom in-house software to generate splicing events including exon skipping, novel intron insertion and alternative 5′ donor and 3′ acceptor splicing sites.

SUMMARY OF RESULTS

- In Figure 1 we show a representative example of the analysis performed on LCLs. RT-PCR products of the indicated BRCA1 mutation along with 2 control LCLs were analyzed in triplicate by TapeStation (Fig. 1A shows two representative replicates), CE (Fig. 1B), and by cloning followed by Sanger sequencing analysis (Fig. 1C). We were able to detect and characterize the splicing aberrations described in the literature by the ENIGMA consortium (4), in addition to novel transcripts not previously reported (Table 1).
- In Table 2 we demonstrate that our RT-PCR RNA-Seq protocol is able to perform *quantitative* and *qualitative* characterization of splicing defects. RT-PCR RNA-Seq was also able to identify novel transcripts not previously detected indicating it has higher sensitivity and specificity than the current gold-standard.
- In Figure 2 we provide the example of the RNA Studies that lead to the reclassification of BRCA1 c.5152+5G>T from VUS to likely-pathogenic (VLP). Family Studies (Fig 2A), gel analysis (Fig 2B), CE analysis (Fig 2C), cloning and Sanger (Fig 2D), and RT-PCR RNA-Seq (Fig 2E) demonstrated that the variant causes mostly in skipping of exon 17 (△17). Skipping of the 78 nucleotides of exon 17 results in the retention of the same reading frame and in the removal of 26 amino acids, disrupting the first BRCT (BRCA1 C- terminus) domain of BRCA1.

RNA Studies Validation



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	Variant puro	Contorl 1 puro	Control2 puro	Variant CHX	Contorl 1 CHX	Control2 CHX
% Abnormal Round 1	50	5	5	59	16	12
% Abnormal Round 2	70	18	15	67	29	26

FIGURE 1: **Validation of Ambry's RNA Studies protocol.** A) TapeStation gel analysis showing two independent RT-PCRs detecting the previously described alternative spliced isoform lacking exon 23 (△23). B) CE showing the described alternative spliced isoform lacking exon 23 (△23) is highly expressed in LCL BRCA1 c.5467+5G>T, but not in controls LCLs. C) Summary of cloning Sanger results indicating the percentage of abnormal transcripts identified in LCL BRCA1 c.5467+5G>T and control LCLs. Puro (Puromycin) and CHX (cyclohexamide) are Nonsense Mediated RNA Decay (NMD) inhibitors.

BRCA1c.5467+5G>T		Detected by TapeStation - Variant	Detected by TapeStation - Controls	Detected by CE-Variant	Detected by CE-Controls	Detected by Sanger - Variant	Detected by Sanger - Controls
Full-length		1,2,3	1,2,3	1,2,3	1,2,3	1	1
Δ21	c.5278_5332del55			3		2	1,2
Δ22	c.5333_5406del74						
Δ23	c.5407_5467del61	1,2,3	2,3	1,2,3*		1,2*	
Δ21,23	c.5278_5332del56_c.5407_5467del61						
Δ22,23	c.5333_5467del135	2*					
ins i20	c.5277+2644_5277+2735ins92						1,2
ins i20	c.5278-391_5278-134ins258						2
ins i20	c.5278-51_5278-1ins51						2
ins i21	c.5332+873_5333-868ins129						1
ins i21	c.5332+873_5333-588ins409					2	2
ins i22	c.5407-9_5407-1ins9						1,2
ins i23	c.5467+1_5467+5ins5						1,2
ins i21 + Δ23	c.5332+873_5333-868ins129_c.5407_5467del61					1*	

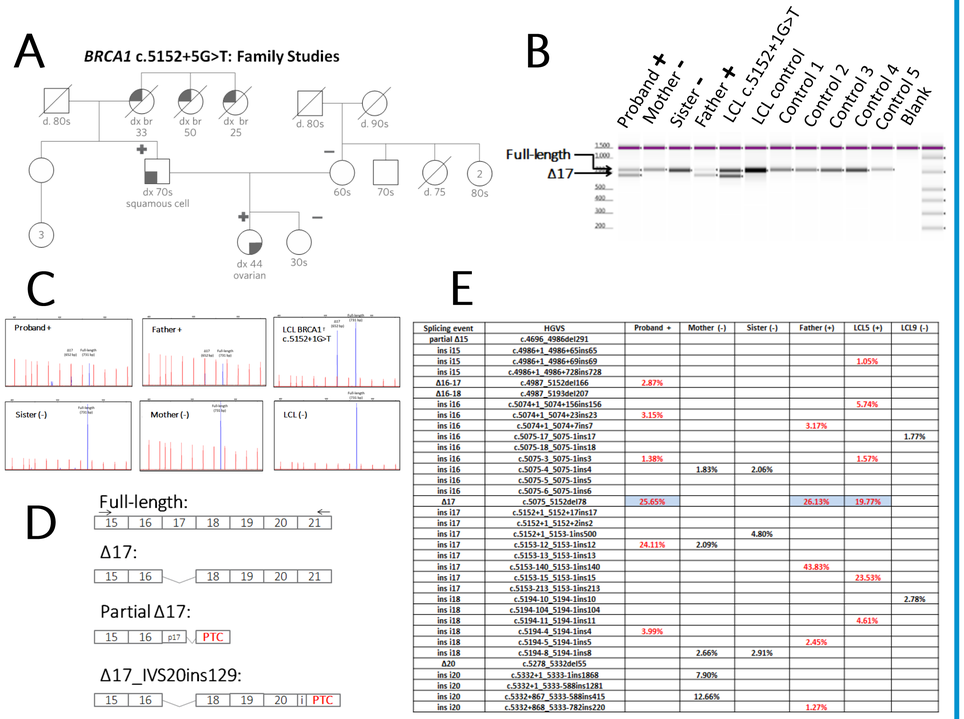
TABLE 1: **Comparison between Ambry and ENIGMA's protocols.** Numbers indicate the detection of the splice variant in replicates 1,2, or 3. * detected in variant only. Shaded = novel transcripts detected by Ambry and not reported by ENIGMA.

RT-PCR RNA-Seq

BRCA1c.5467+5G>T		Variant puro	Contorl 1 puro	Control2 puro	Variant CHX	Contorl 1 CHX	Control2 CHX
Δ21	c.5278_5332del55	2.45%	2.05%	3.15%	3.35%	1.46%	1.94%
Δ22	c.5333_5406del74	1.29%	1.81%	1.33%	2.04%	4.06%	2.01%
Δ23	c.5407_5467del61	45.33%	0.45%	1.27%	41.33%	1.55%	0.38%
Δ22,23	c.5333_5467del135	1.48%	0.34%	1.00%	3.73%	0.34%	1.03%
Δ22,23	c.5423_5498del76	0.03%					
partial Δ23	c.5484_5486del3		0.25%				
ins i20	c.5278-7_5278-1ins7	0.24%	0.36%				
ins i20	c.5278-5_5278-1ins5			0.33%			0.32%
ins i20	c.5277+2644_5277+2735ins92			0.20%			
ins i20	c.5278-391_5278-134ins258						
ins i20	c.5278-51_5278-1ins51				0.41%		
ins i21	c.5333-122_5333-1ins122	0.35%					
ins i21	c.5332+446_5333-124ins1300	1.17%					
ins i21	c.5332+456_5332+844ins389		0.08%				
ins i21	c.5332+847_5333-580ins443		1.20%				
ins i21	c.5332+868_5333-580ins422				0.76%	2.54%	1.30%
ins i21	c.5333-19_5333-1ins19		0.57%		0.38%	0.60%	0.63%
ins i22	c.5407-8_5407-1ins8	0.34%	0.93%	0.78%	0.66%	1.07%	1.31%
ins i22	c.5406+1_5406+4ins4			2.64%		2.83%	
ins i22	c.5406+1_5406+5ins5	1.30%					
ins i22	c.5406+1_5406+6ins6				1.65%		
ins i22	c.5406+1_5406+8ins8						3.02%
ins i22	c.5406+1_5406+13ins13		2.25%				
ins i23	c.5468-7_5468-1ins7		0.07%				
ins i23	c.5467+1_5467+5ins5		0.23%	0.17%		0.10%	
ins i23	c.5467+1_5467+9ins9	0.11%			0.05%		

TABLE 2: **Summary of RT-PCR RNA-Seq results for BRCA1 c.5467+5G>T and control LCLs.** RT-PCR RNA-seq protocol is able to perform *quantitative* and *qualitative* characterization of splicing defects. RT-PCR RNA-Seq was also able to identify novel transcripts not detected by cloning and Sanger sequencing, indicating the protocol has higher sensitivity and specificity compared to the current gold-standard. Red box highlights the most common alternatively spliced transcript (△23) representing 41-45% of the total number of transcripts in LCL BRCA1 c.5467+5G>T, but only 0.38-1.55% of transcripts in control LCLs. Puro (Puromycin) and CHX (cyclohexamide) are NMD inhibitors.

RNA Studies Improves Classification



Splicing event	HGVS	Proband +	Mother (-)	Sister (-)	Father (+)	LCL5 (+)	LCL9 (-)
partial Δ15	c.4696_4986del291						
ins i15	c.4986+1_4986+65ins65						
ins i15	c.4986+1_4986+69ins69					1.05%	
ins i15	c.4986+1_4986+728ins728						
Δ16-17	c.4987_5152del166	2.87%					
Δ16-18	c.4987_5193del207						
ins i16	c.5074+1_5074+156ins156					5.74%	
ins i16	c.5074+1_5074+23ins23	3.15%					
ins i16	c.5074+1_5074+7ins7				3.17%		
ins i16	c.5075-17_5075-1ins17						1.77%
ins i16	c.5075-18_5075-1ins18						
ins i16	c.5075-3_5075-1ins3	1.38%				1.57%	
ins i16	c.5075-4_5075-1ins4		1.83%	2.06%			
ins i16	c.5075-5_5075-1ins5						
ins i16	c.5075-6_5075-1ins6						
Δ17	c.5075_5152del78	25.65%			26.13%	19.77%	
ins i17	c.5152+1_5152+17ins17						
ins i17	c.5152+1_5152+2ins2						
ins i17	c.5152+1_5153-1ins500			4.80%			
ins i17	c.5153-12_5153-1ins12	24.11%	2.09%				
ins i17	c.5153-13_5153-1ins13						
ins i17	c.5153-140_5153-1ins140				43.83%		
ins i17	c.5153-15_5153-1ins15					23.53%	
ins i17	c.5153-213_5153-1ins213						
ins i18	c.5194-10_5194-1ins10						2.78%
ins i18	c.5194-104_5194-1ins104						
ins i18	c.5194-11_5194-1ins11					4.61%	
ins i18	c.5194-4_5194-1ins4	3.99%					
ins i18	c.5194-5_5194-1ins5				2.45%		
ins i18	c.5194-8_5194-1ins8		2.66%	2.91%			
Δ20	c.5278_5332del55						
ins i20	c.5332+1_5333-1ins1868		7.90%				
ins i20	c.5332+1_5333-588ins1281						
ins i20	c.5332+867_5333-588ins415		12.66%				
ins i20	c.5332+868_5333-782ins220				1.37%		

Figure 2: **RNA Studies of BRCA1 c.5152+5G>T lead to VUS to VLP reclassification.** A) Family history. B) TapeStation. C) CE. D) Scheme of transcripts detected by cloning and Sanger. E) RT-PCR RNA-seq.

TAKE-HOME POINTS

- Our RNA Studies protocol was validated using ENIGMA's gold-standard protocol.
- RNA Studies allow accurate classification of splicing variants, reducing VUS classifications.
- RNA-NGS provides a sensitive and specific high-throughput alternative to the gold-standard, thereby improving the interpretation of splicing variants detected on clinical genomic tests.

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