Title:

RNA-based Approach Identifies Pathogenic Tandem Duplications in Hereditary Cancer Genes

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Description:

BACKGROUND: Current methods to detect gross duplications in genomic DNA (MLPA, aCGH, NGS) are complicated by a number of factors, including: (i) failure to identify the precise location of copy number (CN) gains in the genome; (ii) ambiguity generated by the presence of pseudogenes; and (iii) inability to evaluate whether the gross duplication results in abnormal transcripts. Due to these issues and the lack of specificity of current detection methods, duplications are often reported as variants of unknown significance (VUS), causing confusion for clinicians and anxiety for patients. With these issues in mind, an RNA based technique – *Tandem RT-PCR* – was applied to bypass the issues inherent to gDNA methods. This Tandem RT-PCR assay facilitated identification of abnormal transcripts associated with tandem duplications.

METHODS: The following duplications were examined: PTEN EX3_6dup, CHEK2 EX2_6dup, MSH2 EX14dup, and PMS2 EX9dup. Briefly, total RNA was isolated from whole blood (PAXgene, Qiagen) and cDNA synthesized with 500ng input from mRNA (SuperScript IV, Invitrogen). Sense and antisense primers were designed on cDNA in the reverse orientation within the duplicated region, requiring a tandem duplication to create an amplicon (Tandem RT-PCR). Primers were also designed in the proper orientation (RT-PCR) in exons flanking the duplicated region. Both primer sets were run under the same conditions (500ng cDNA, 0.5uM primer, HotstarTaq with 1.5mM $MgCl_2$, 35 cycles with T_a of 60°C and 45s elongation). Amplicons were quantified (TapeStation 2200, Aglient) and Sanger sequenced to confirm presence of abnormal exon junctions (20ng Tandem RT-PCR product, 2.5uM primer; Genewiz). Resulting electropherograms were aligned manually to reference cDNA sequence (GRCh37: PTEN NM_000314, CHEK2 NM_007194, MSH2 NM_000251, PMS2 NM_000535).

RESULTS: RT-PCR produced bands in proband and controls of the expected size, confirming PCR conditions are suitable to create amplicons in the duplicated region in all genes assayed. Tandem RT-PCR produced the specific band only in the carriers of the PTEN, CHEK2 and MSH2 duplications. For PTEN, Sanger sequencing confirmed

abnormal EX6_EX3 junction; for CHEK2, sequencing revealed the abnormal EX6_EX2 junction; while MSH2 revealed an abnormal EX14_EX14 junction. PMS2 was a unique case as the Tandem RT-PCR failed to produce a band in the proband, and RT-PCR amplified the normal transcript but did not produce the expected duplicated transcript in the proband. Sanger sequencing of gDNA identified a PMS2CL gene conversion in the sequence analogous to intron 9 of PMS2 that was allowing MLPA probes in that region to ligate and amplify, causing an apparent CN gain.

CONCLUSION: The RNA-based Tandem RT-PCR assay was capable of characterizing duplications in PTEN, CHEK2 and MSH2 as intragenic, in tandem and out of frame. PTEN, CHEK2, MSH2, are hot spots for such duplications due to presence of Alu elements and other such retrotransposons (Qian et al 2017). With the evidence obtained by Tandem RT-PCR, the intragenic tandem duplications in these cancer genes were reclassified from VUS to likely pathogenic, making them clinically actionable. This Tandem RT-PCR assay was also able to rule out PMS2 EX9dup that was instead the result of a gene conversion. Using RNA for identification of gross duplications alleviates the need to determine deep intronic breakpoints for which traditional gDNA assays would need custom probes and primers. MLPA and aCGH are not specific enough to determine the chromosomal location of CN gains and are complicated by the presence pseudogenes and gene conversions. Assaying mRNA simplifies issues associated with pseudogenes, especially in PMS2, since most are not polyadenylated (Etzler et al 2008). In conclusion, the Tandem RT-PCR assay facilitated classification of duplications that were originally reported as VUS in a timely manner, providing clinicians with reliable evidence about the cause of their patient's disease that will help inform preventive measures and treatment.

Keywords:
Chromosomal Abnormalities
Genetic Testing
Genomic Methodologies
Methodology
Mutation Detection
Sequencing

Primary Topic Focus:
Cancer Genetics

Learning Objective 1:	The audience will be able to describe the benefits of using RNA-based assays to identify tandem duplications