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

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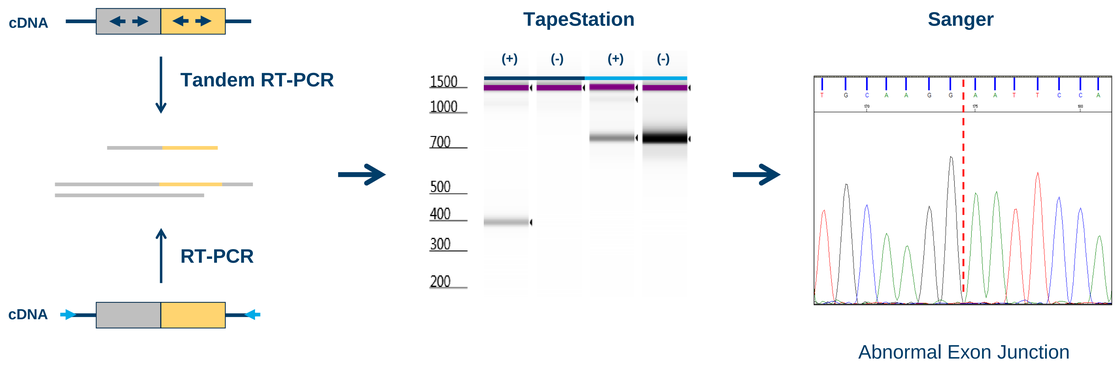
## BACKGROUND

- Germline duplications are classified as pathogenic if:
  - Proven exonic and intragenic
  - Confirmed In tandem
  - Shown to cause frame shift
- Usually, gDNA methods fail to provide chromosomal location of duplications via deep intronic breakpoints, resulting in the alteration being reported as variant of unknown significance (VUS). This causes confusion for caregivers and anxiety for patients.
- Hereditary cancer genes are hot spots for duplications arising from Alu mediated intrastrand slipping-mispairing during double stranded DNA repair [1].
- RNA-based approach provides functional evidence where gDNA methods are lacking. This evidence can be used to classify the alterations as likely-pathogenic (LP).

## TANDEM RT-PCR WORKFLOW




## METHODS

- RNA isolation: Patient's whole blood was collected in PAXgene tubes containing RNA stabilizers. Total RNA was isolated using PAXgene kit (Qiagen).
- cDNA synthesis: Superscript IV (Invitrogen) using oligo-dT to select for mRNA.
- Primer design: All primers designed with similar thermodynamic properties on cDNA refseq (GRCh37: *PTEN* NM_000314, *CHEK2* NM_007194, *MSH2* NM_000251). Tandem RT-PCR: primers in opposite orientation within duplicated region. RT-PCR: primers in traditional orientation flanking duplicated region.
- PCR Conditions: 500ng cDNA, 0.5uM primer, HotstarTaq with 1.5mM $MgCl_2$, 35 cycles with $T_a$ of 60°C and 45s elongation.
- Sanger sequencing: 20ng Tandem RT-PCR product, 2.5uM primer (Genewiz).
- Cloning: 12ng RT-PCR product was A-tailed, ligated into pGEM-T, and transformed using pGEM-Teasy kit (Promega). Colony PCR was performed using individual colonies as template with the above conditions. Then, colony PCR product was Sanger sequenced using the above conditions.

## Figure 1: *PTEN*

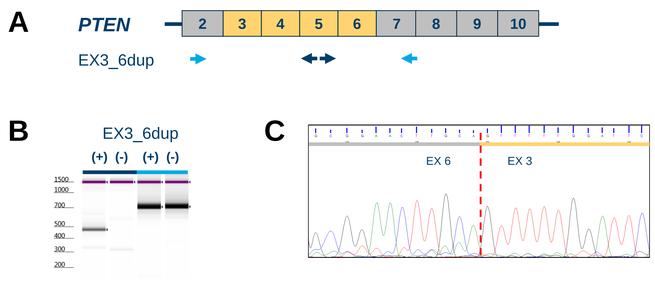



**Figure 1**: Tandem RT-PCR for a *PTEN* duplication. A. Schematic diagram of CDS, with exons involved in duplications (yellow), RT-PCR (light blue) and Tandem RT-PCR (dark blue) primer design; B. TapeStation image of Tandem RT-PCR (dark blue) and RT-PCR products (light blue) for proband (+) and WT blood control (-); C. Sanger sequencing of Tandem RT-PCR product present in proband only confirms abnormal exon junction in the forward read.

## Figure 2: *MSH2*

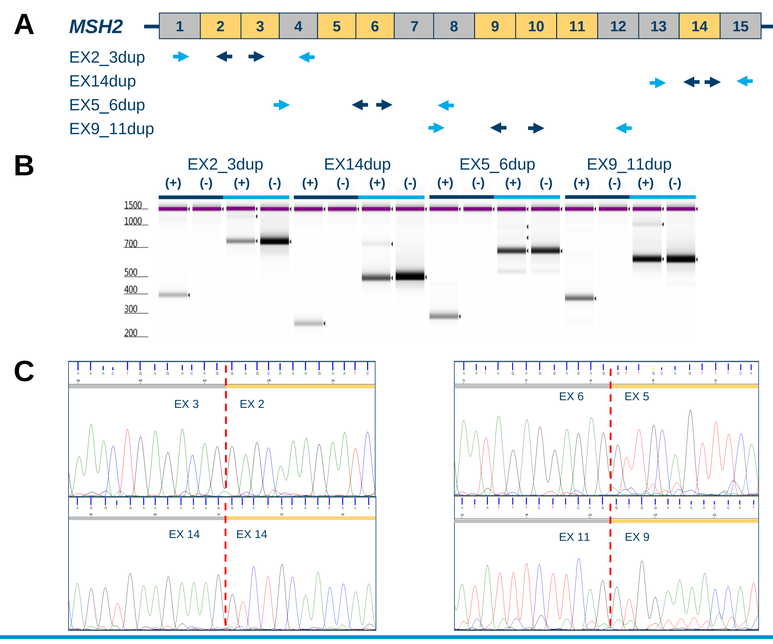



**Figure 2:** Tandem RT-PCR for duplications in *MSH2*. A. Schematic diagram of CDS, with exons involved in duplications (yellow), RT-PCR (light blue) and Tandem RT-PCR (dark blue) primer design; B. TapeStation image of Tandem RT-PCR (dark blue) and RT-PCR products (light blue) for proband (+), WT blood control (-), and WT tissue control (-) Colon; C. Sanger sequencing of Tandem RT-PCR product present in probands only confirms abnormal exon junction in the forward read.

## Figure 3: *CHEK2*

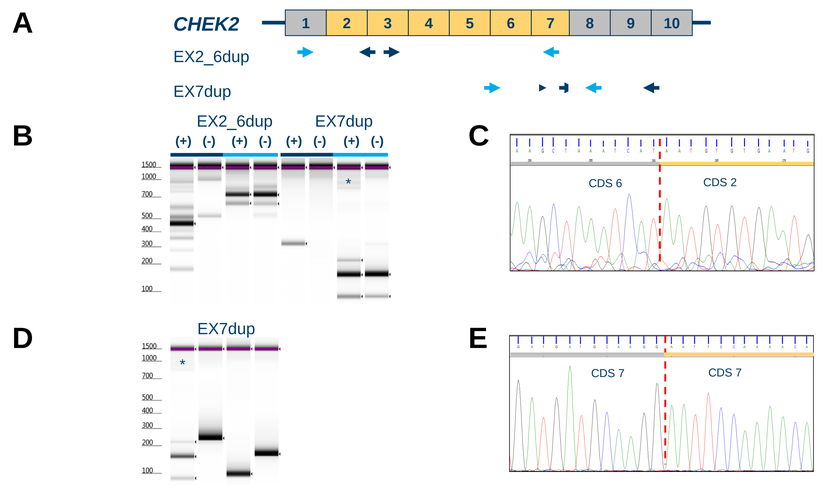



**Figure 3**: Tandem RT-PCR for duplications in *CHEK2*. A. Schematic diagram of CDS, with exons involved in duplications (yellow), RT-PCR (light blue) and Tandem RT-PCR (dark blue) primer design; B. TapeStation image of Tandem RT-PCR product (dark blue) and RT-PCR product (light blue) of proband (+) and WT blood control (-); C. Sanger sequencing of CHEK2 EX2_6dup Tandem RT-PCR product; D. Colony PCR of clones transformed with RT-PCR product (*) from the EX7dup proband; E. Sanger sequencing of highest molecular weight colony PCR product.

## RECLASSIFICATION FROM VUS → LIKELY PATHOOGENIC (LP)

| Variant | Clinical History | Family History | Classification After DNA Analysis | Classification After RNA Analysis | NCCN Guidelines |
|---|---|---|---|---|---|
| ***PTEN* EX3_6dup** | Multiple colon adenomas and single PJS polyp at age 58 | Father: colon resection at age 40 | VUS | LP | Annual thyroid ultrasound, colonoscopy, biennial renal imaging [2] |
| ***MSH2* EX2_3dup** | Sigmoid colon adenocarcinoma at age 10 | Paternal: distant history of colon cancer<br>Maternal grandmother: breast cancer at age 54 | VUS | LP | Annual colonoscopy, potential prophylactic surgery [3] |
| ***MSH2* EX14dup** | Female diagnosed with endometrial cancer at age 53 and ileocecal adenocarcinoma at age 63 | Sister: endocervical carcinoma at age 43<br>Parents: colon cancer at age 60 | VUS | LP | Annual colonoscopy, potential prophylactic surgery [3] |
| ***MSH2* EX5_6dup** | 17 colon TA polyps and 4 fundic polyps at age 39 | Brother: history of colon polyps<br>Maternal cousin: breast and uterine cancer | VUS | LP | Annual colonoscopy, potential prophylactic surgery [3] |
| ***MSH2* EX9_11dup** | Right colon adenocarcinoma at age 44 | Maternal grandmother: colon cancer at age 86<br>Maternal uncle: colon cancer at ages 24 and 70 | VUS | LP | Annual colonoscopy, potential prophylactic surgery [3] |
| ***CHEK2* EX2_6dup** | Ovarian adenocarcinoma at age 56 | Father: prostate cancer at unknown age<br>Paternal niece: tongue cancer at age 40 | VUS | LP | Annual mammogram, MRI with contrast, potential prophylactic mastectomy [4] |
| ***CHEK2* EX7dup** | No cancer at age 47 | Mother: breast cancer at age 70<br>Maternal aunt: breast cancer at age 65<br>Maternal aunt: breast cancer at age 65 | VUS | LP | Annual mammogram, MRI with contrast, potential prophylactic mastectomy [4] |

## TAKE-HOME POINTS

- Tandem RT-PCR provides functional evidence allowing proper classification of germline duplications in a cost and time efficient manner.
- RNA-based functional evidence provides:
  - Confirmation duplication is in tandem
  - Proof of abnormal splicing
  - Accurate calculation of frame shift
- For medical providers, when a VUS duplication is reclassified to LP, it is more clear as to how to manage the patient.
- For the patients, reclassification results in less anxiety and more clarity when managing cancer risks for themselves and family members.

## REFERENCES

1. Qian et al. Cancer Genet. 2017; 216-217:159-169
2. NCCN Clinical Practice Guidelines in Oncology®. Genetic/Familial High-Risk Assessment: Breast and Ovarian. V 2.2016.
3. NCCN Clinical Practice Guidelines in Oncology®. Genetic/Familial High-Risk Assessment: Colorectal. V.3.2017.
4. NCCN Clinical Practice Guidelines in Oncology®. Genetic/Familial High-Risk Assessment: Breast and Ovarian. V1.2017.