THE LANCET
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## Supplementary appendix

This appendix formed part of the original submission and has been peer reviewed. We post it as supplied by the authors.

Supplement to: Fewings E, Larionov A, Redman J, et al. Germline pathogenic variants in *PALB2* and other cancer-predisposing genes in families with hereditary diffuse gastric cancer without *CDH1* mutation: a whole-exome sequencing study. *Lancet Gastroenterol Hepatol* 2018; published online April 26. http://dx.doi.org/10.1016/S2468-1253(18)30079-7.

# Supplementary material: Whole exome sequencing study to detect germline pathogenic variants in *PALB2* and other cancer-predisposing genes in *CDH1*-negative diffuse gastric cancer families.

## Supplementary Materials and Methods:

### Bioinformatics pipeline for VCF generation

Fastq files underwent demultiplexing and standard QC checks using FastQC prior to trimming of Illumina adaptors and low quality bases using Cutadapt (ver 1.8.1). The BWA-MEM algorithm (ver 0.7.12) was applied to align reads to GRCh37. BAM files from multiple lanes were merged, sorted and pre-processed (removal of PCR duplicates, base quality recalibration and local realignment around indels) using Samtools (ver 1.2), Picard (ver 2.6.0) and GATK (ver 3.6.0). Variant calling was performed across the set with GATK Haplotype Caller with 10bp padding around Nextera Exome Rapid Capture targets.

Optimised hard filters were applied, including a VQSR truth sensitivity of 99·5% for SNPs and 97% for INDELs, an average 10x depth (variant DP) per sample and a QUAL threshold of 200. The QUAL threshold corresponded to a TiTv ratio of 2 as calculated by Samtools VCF-Stats. Multi-allelic variants were flagged and excluded for the purpose of this analysis. Only genotypes with quality (GQ) >20 and individual depth (genotype DP) in sample < 500 were retained for further analysis. Ensembl VEP annotations were applied to select protein-affecting variants: loss of function (stop gained, stop lost, start lost, splice acceptor variant, splice donor variant, or frameshift variant), inframe indels and missense variants that were simultaneously called deleterious and probably damaging by SIFT and PolyPhen respectively. Common variants (AF > 0.05 in European 1000 genomes) were excluded. The non-common protein-affecting variants were aggregated per gene; these genes were used for interaction analyses and prioritised as described in main methods and in scripts below.

Scripts generated for all analysis downstream of VCF generation can be found at the following link (https://github.com/elliefewings/Fewings_HDGC_exome_2018). VCF data can be downloaded from the following repository (https://doi.org/10.17863/CAM.17181)

### Validation by Sanger sequencing

Custom primers were designed for each variant and are summarised in supplementary table 1. Primers were designed to be between 18 and 26 bases in length with a melting temperature of around 60°C. The UCSC In-Silico PCR tool was used to check specificity of primer binding. Due to their proximity, both *RECQL5* variants (c.2806-2T>C and c.2828C>T) were covered by one pair of primers.

### Gene interaction network analysis – Control data

The 1000 genomes project was used as a control set to test for an enrichment of loss of function variants under selected gene ontology terms in HDGC. Variants from European phase-3 1000 genomes data were filtered to select 28,833 uncommon (European AF <0·05 in 1000 genomes), protein affecting variants (loss of function, predicted deleterious and damaging missense and inframe indels). Variants were aggregated into 11,796 genes, which were filtered to select those with at least one loss of function variant and remove the top 1% most variable genes. Variability was measured by the number of rare, protein affecting variants each gene contains; 3,634 genes containing 4,601 loss of function variants were retained. Aggregated allele counts for each selected gene ontology term were generated using these loss of function variants for further analysis.

## Supplementary Results:

### VCF generation and quality metrics

Samples were sequenced across five whole exome sequencing libraries. Data quality of aligned, merged BAM files was checked using metrics generated by Qualimap and Picard (supplementary table 3). The mean percentage of targets covered at 20x across all samples was 80.23%. All identified candidate variants were manually checked in BAM files using IGV for region coverage and appropriate percentage of reads supporting the alternative variant call. Additionally all candidate variants were validated successfully by Sanger sequencing.

## Supplementary Tables and Figures:

| Gene | Variant | Forward Primer 5’-3’ | Reverse Primer 5’-3’ |
|---|---|---|---|
| *PALB2* | c.757-758TAG>T | GGAGAGAGACTGTGTCTTTGGCACTG | AGAGGTTGCTTCCAGGCTAAGACTC |
| *RECQL5* | c.2806-2T>C<br>and<br>c.2828C>T | CGTGTTAGCCAGGATGGTCTCG | CATGAGGAGGTGAGCGTTAGCC |
| *MSH2* | c.967-968T>TCTCA | GCGGGGCTTAGTGGCGTG | GACATCGCACCCAGCCCC |
| *ATR* | c.6075A>T | CCATTGATGTGGAACCTGTGGCTAC | GATTACTGGGATGAAGGGTAGTGGGG |
| *NBN* | c.1123+1C>G | CCCGTCATAGATGCCCGCAG | GCAGAGTGGAGGAGCTGGGAC |
| *MSH2* | c.1A>C | ACCTGGTGGCAACCTACCCTTG | ACCCCCTGGGTCTTGAACACC |

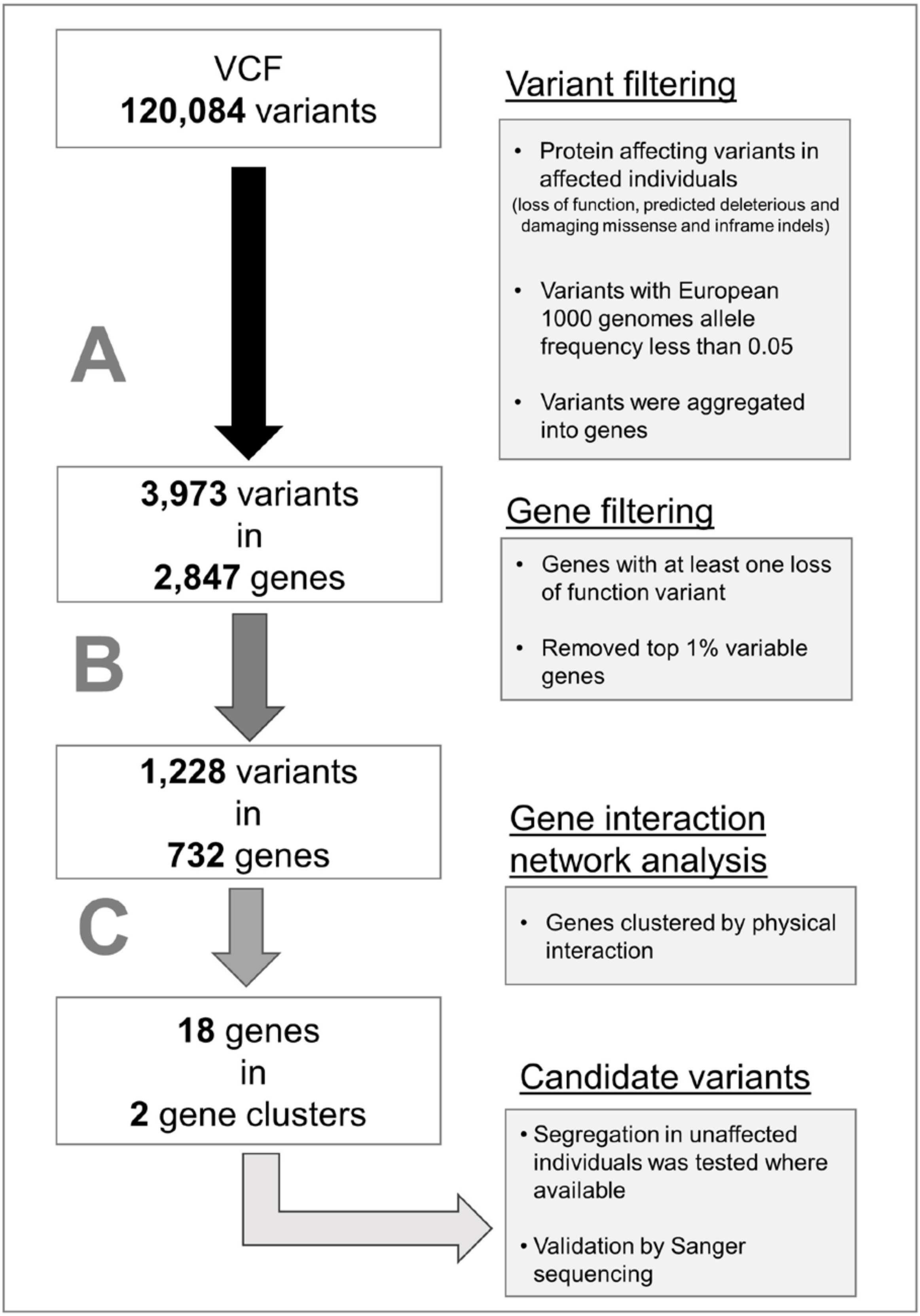
**Supplementary table 1:** Primers used to validate candidate variants by Sanger sequencing.

| GO Term | Counts for minor alleles in HDGC | Counts for minor alleles in Controls | Counts for major alleles in HDGC | Counts for major alleles in Controls | One-tailed Fishers Exact P value |
|---|---|---|---|---|---|
| Double strand break repair (GO:0006302) | 13 | 118 | 31 | 888 | 0·0005 |
| Negative regulation of extrinsic apoptotic signaling pathway via death domain receptors (GO:1902042) | 4 | 50 | 40 | 956 | 0·186 |

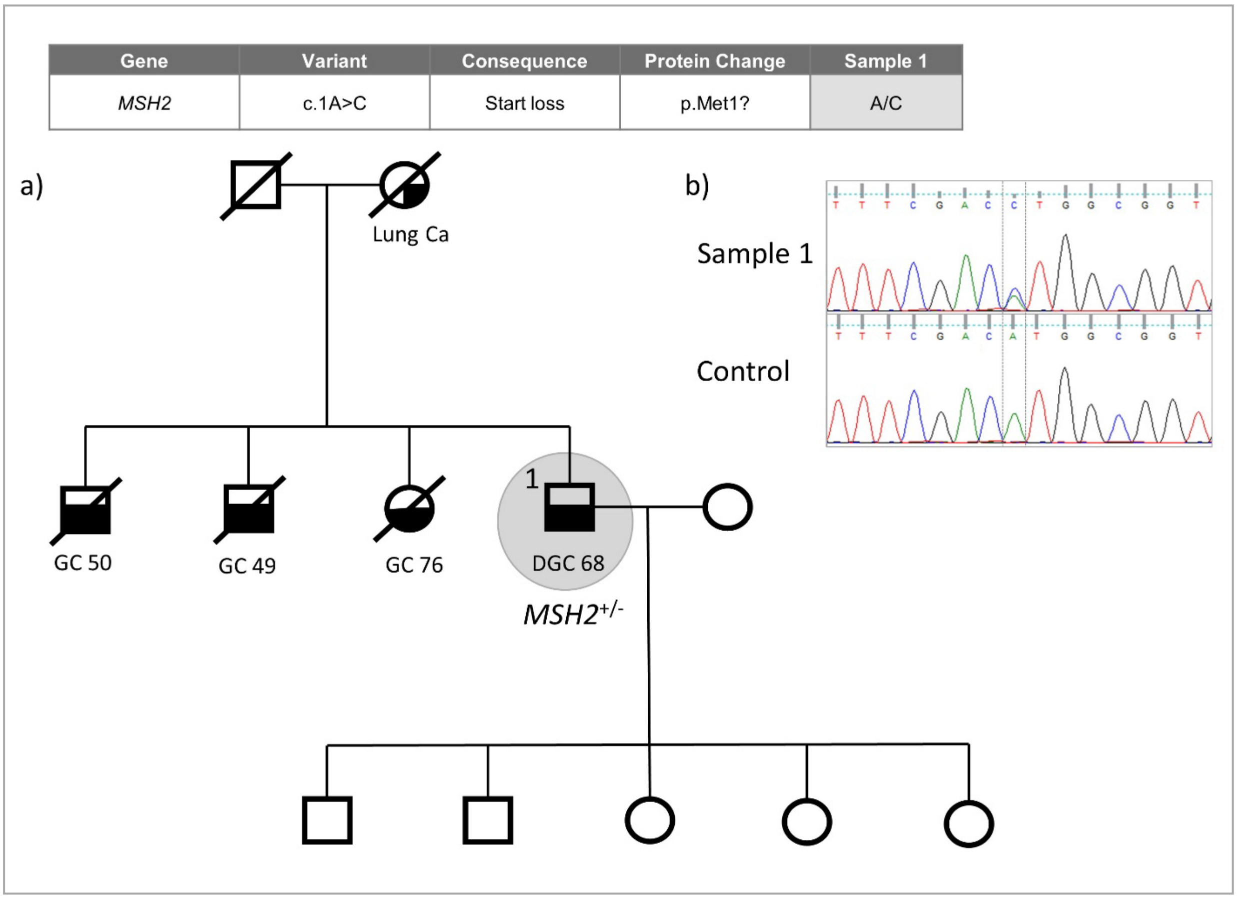
**Supplementary table 2:** Aggregated allele counts of non-common protein-affecting variants within the HDGC set and a European 1000 genomes control set.

| Sample | Percentage Mapped Reads | Mean Insert Size | Mean Mapping Quality | GC% | Mean Coverage on targets (X) | Percentage at 20X Coverage on targets |
|---|---|---|---|---|---|---|
| GPQ_045_202 | 99.96% | 170.63 | 57.42 | 48.58% | 35.25 | 72.16% |
| GPQ_045_203 | 99.97% | 150.41 | 57.37 | 48.64% | 25.25 | 56.65% |
| GPQ_047_301 | 99.89% | 185.51 | 57.07 | 48.60% | 76.08 | 85.63% |
| GPQ_047_302 | 99.89% | 182.58 | 57.06 | 47.71% | 62.05 | 81.03% |
| GPQ_047_303 | 99.76% | 100.68 | 56.76 | 48.61% | 40.37 | 72.55% |
| GPQ_047_304 | 99.93% | 151.03 | 57.06 | 48.04% | 56.07 | 80.67% |
| GPQ_047_305 | 99.94% | 133.57 | 57.02 | 46.80% | 51.75 | 75.47% |
| GPQ_047_308 | 99.67% | 107.89 | 56.78 | 49.33% | 29.81 | 63.63% |
| GPQ_048_401 | 99.98% | 184.18 | 57.43 | 48.89% | 50.76 | 84.36% |
| GST_172_301 | 99.97% | 198.75 | 57.40 | 49.24% | 47.21 | 83.18% |
| GST_172_302 | 99.96% | 180.50 | 57.36 | 48.91% | 65.33 | 89.33% |
| GST_172_303 | 99.97% | 146.01 | 57.36 | 48.39% | 33.32 | 68.88% |
| GST_230_304 | 99.98% | 148.69 | 57.35 | 53.37% | 43.62 | 70.30% |
| GST_256_301 | 99.97% | 176.90 | 57.43 | 48.48% | 64.44 | 88.54% |
| GST_257_201 | 99.97% | 205.06 | 57.41 | 53.43% | 71.48 | 87.47% |
| GST_257_202 | 99.98% | 118.26 | 57.26 | 50.38% | 32.14 | 64.91% |
| GST_275_201 | 99.96% | 194.56 | 57.44 | 49.31% | 69.97 | 91.53% |
| GST_296_201 | 99.96% | 207.70 | 57.47 | 49.19% | 51.99 | 85.58% |
| GST_345_301 | 99.96% | 136.39 | 57.33 | 48.49% | 59.64 | 84.49% |
| GST_345_302 | 99.97% | 208.28 | 57.47 | 49.23% | 96.14 | 94.73% |
| GST_345_303 | 99.97% | 197.11 | 57.44 | 49.34% | 87.28 | 93.73% |
| GST_349_202 | 99.95% | 117.72 | 57.38 | 47.74% | 101.16 | 87.64% |
| GST_349_252 | 99.92% | 176.85 | 57.05 | 46.74% | 77.56 | 84.28% |
| GST_349_301 | 99.91% | 164.34 | 57.08 | 46.68% | 66.37 | 81.09% |
| GST_349_302 | 99.96% | 154.59 | 57.12 | 46.53% | 45.50 | 73.69% |
| GST_349_303 | 99.94% | 158.02 | 57.03 | 47.29% | 51.10 | 76.71% |
| GST_358_301 | 99.97% | 142.11 | 57.27 | 49.71% | 54.06 | 83.55% |
| GST_368_301 | 99.96% | 213.02 | 57.41 | 49.58% | 141.41 | 97.46% |
| GST_440_403 | 99.97% | 177.69 | 57.37 | 49.01% | 76.64 | 91.39% |
| GST_441_301 | 99.95% | 117.92 | 57.38 | 47.72% | 96.99 | 87.57% |
| GST_444_301 | 99.96% | 151.16 | 57.12 | 47.72% | 48.18 | 77.44% |
| GST_446_301 | 99.93% | 161.09 | 57.07 | 49.59% | 21.04 | 45.64% |
| GST_455_301 | 99.96% | 169.64 | 57.10 | 48.88% | 43.94 | 76.84% |
| GST_459_301 | 99.92% | 198.13 | 57.14 | 49.61% | 96.14 | 90.30% |
| GST_459_302 | 99.93% | 182.49 | 57.10 | 48.33% | 79.52 | 87.00% |
| GST_460_201 | 99.94% | 174.23 | 57.13 | 48.97% | 53.45 | 81.35% |
| GST_463_301 | 99.91% | 174.42 | 57.06 | 48.18% | 54.02 | 81.95% |
| GST_463_402 | 99.95% | 123.09 | 56.98 | 47.44% | 28.85 | 58.78% |
| GST_464_301 | 99.94% | 137.65 | 57.48 | 48.05% | 129.07 | 91.63% |

**Supplementary table 3:** Quality metrics generated from aligned and merged BAM files


VCF
120,084 variants
A
3,973 variants
in
2,847 genes
B
1,228 variants
in
732 genes
C
18 genes
in
2 gene clusters
Variant filtering
• Protein affecting variants in affected individuals
(loss of function, predicted deleterious and damaging missense and inframe indels)
• Variants with European 1000 genomes allele frequency less than 0.05
• Variants were aggregated into genes
Gene filtering
• Genes with at least one loss of function variant
• Removed top 1% variable genes
Gene interaction network analysis
• Genes clustered by physical interaction
Candidate variants
• Segregation in unaffected individuals was tested where available
• Validation by Sanger sequencing

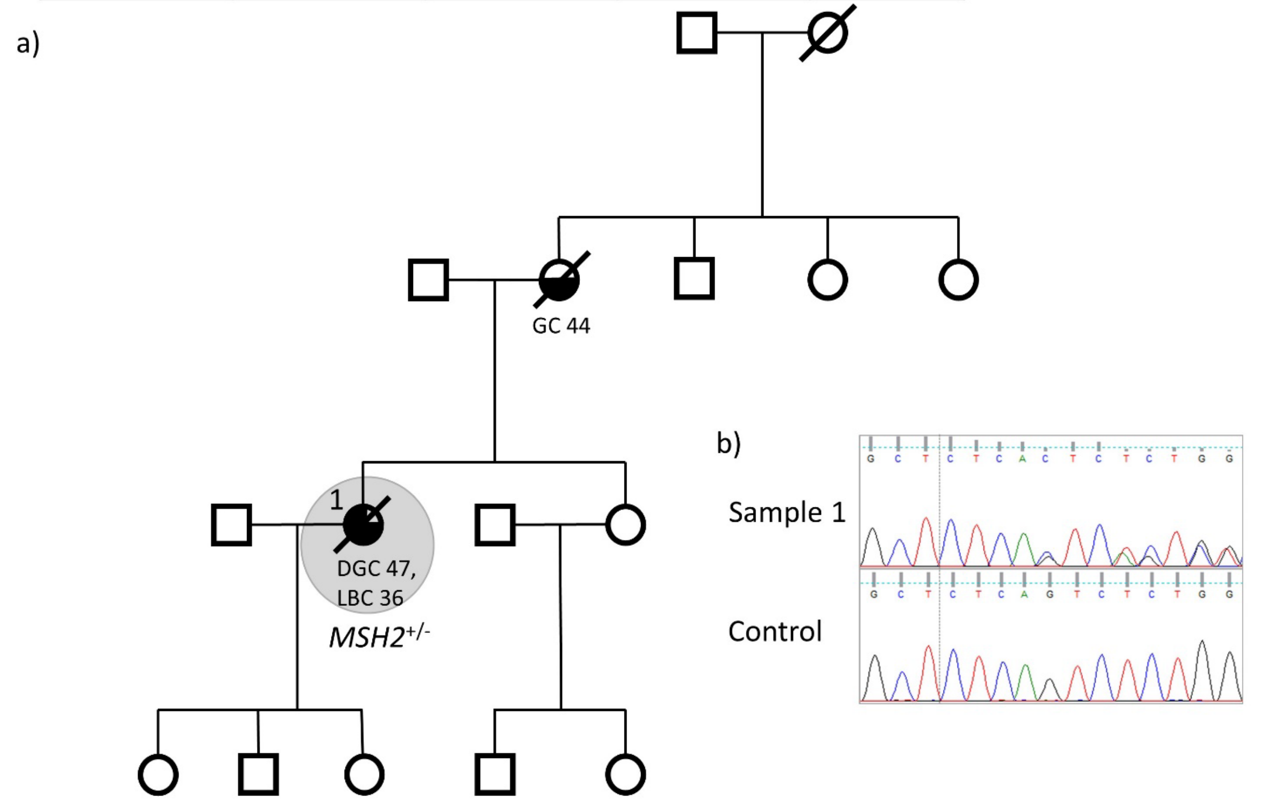
**Supplementary figure 1**: Variants filtering and analysis. A) variant filtering, B) gene filtering and C) gene clustering.



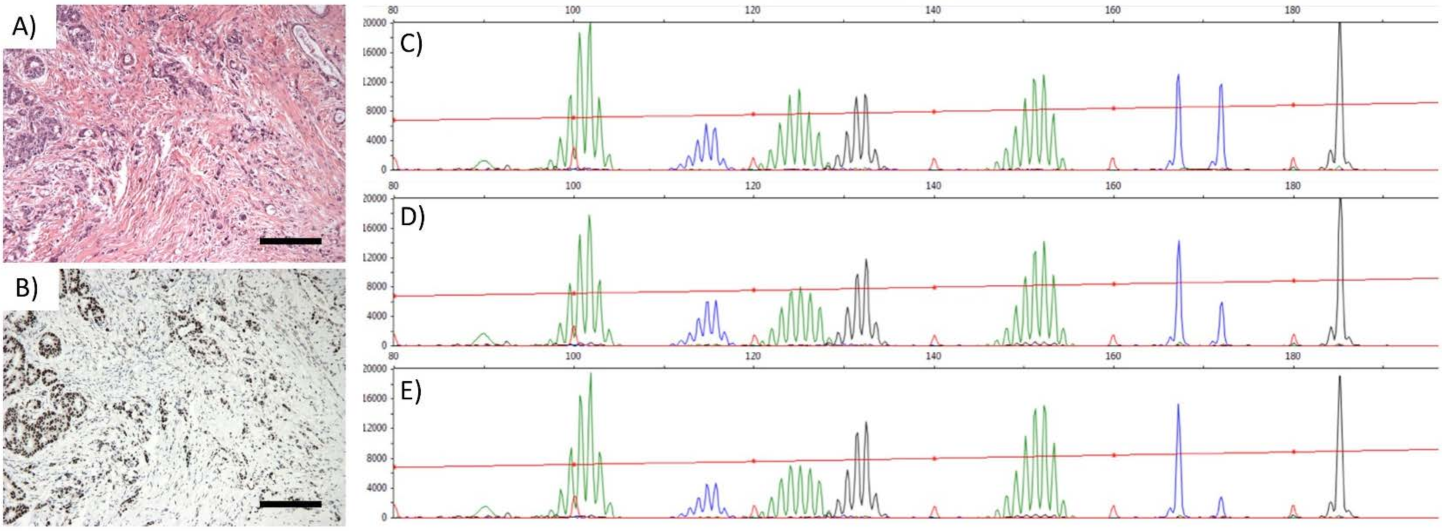
| Gene | Variant | Consequence | Protein Change | Sample 1 |
|---|---|---|---|---|
| *MSH2* | c.1A>C | Start loss | p.Met1? | A/C |

**Supplementary Figure 2**: a) The pedigree for family 12. b) Chromatograms showing the *MSH2* start loss variant in the proband against control DNA. Whole exome sequencing was performed on the circled sample, where shading indicates an affected individual.

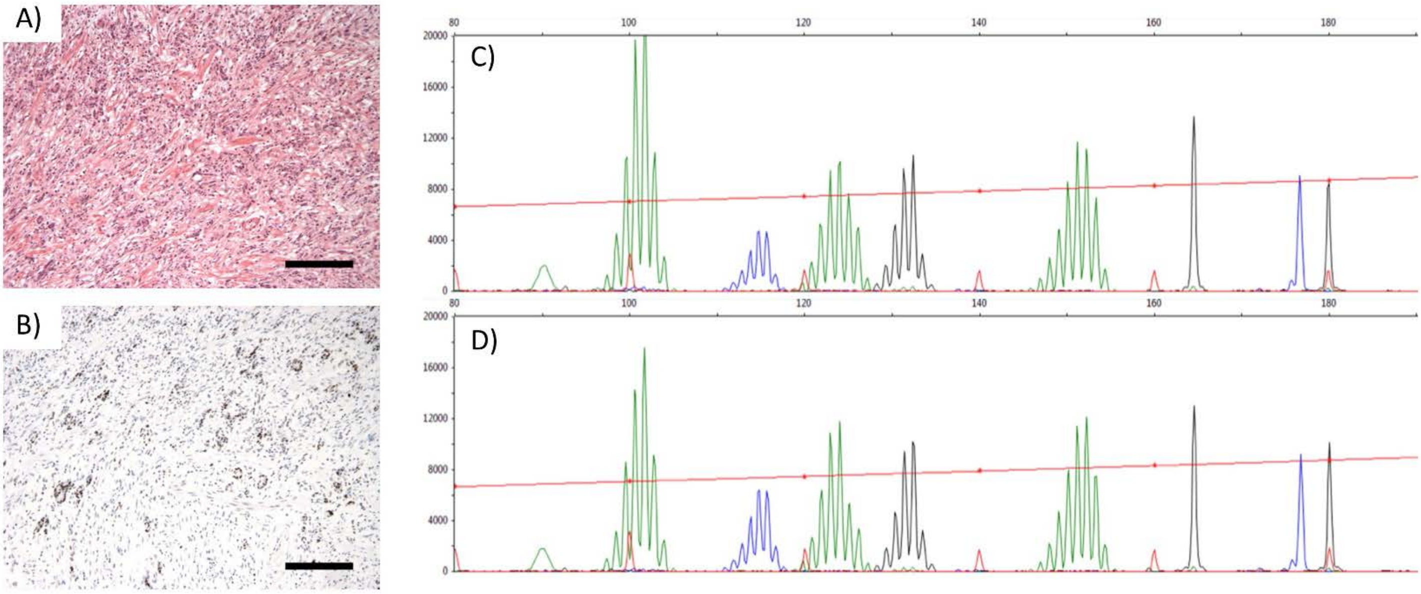
| Gene | Variant | Consequence | Protein Change | Sample 1 |
|---|---|---|---|---|
| *MSH2* | c.967-968T>TCTCA | Frameshift variant | p.Ser323fs | T/TCTCA |



**Supplementary Figure 3**: a) The pedigree for family 8. b) Chromatograms showing the *MSH2* frameshift variant in the proband against control DNA. Whole exome sequencing was performed on the circled sample, where shading indicates an affected individual.

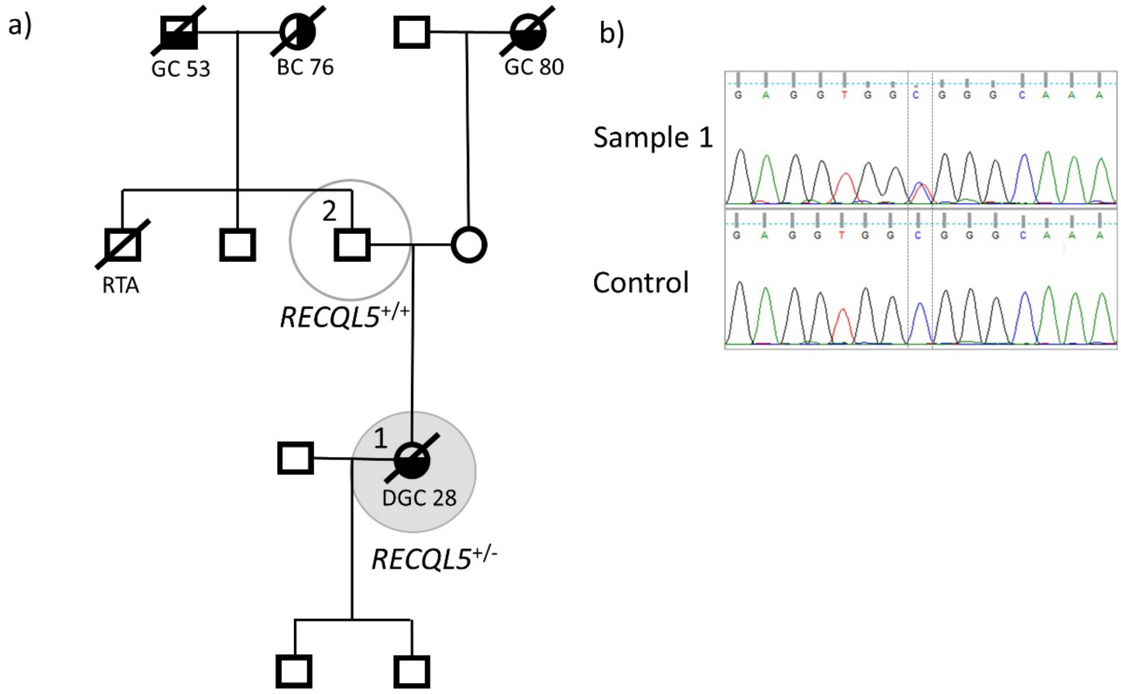


**Supplementary Figure 4:** Family 12 tumour analysis showing representative photomircographs (scale bar 0.2mm) showing a) hematoxylin and eosin b) normal MSH2 expression in tumours. Microsatellites are comparable across c) tumour-free adjacent tissue d) moderately differentiated gastric cancer tissue e) poorly differentiated gastric tissue.



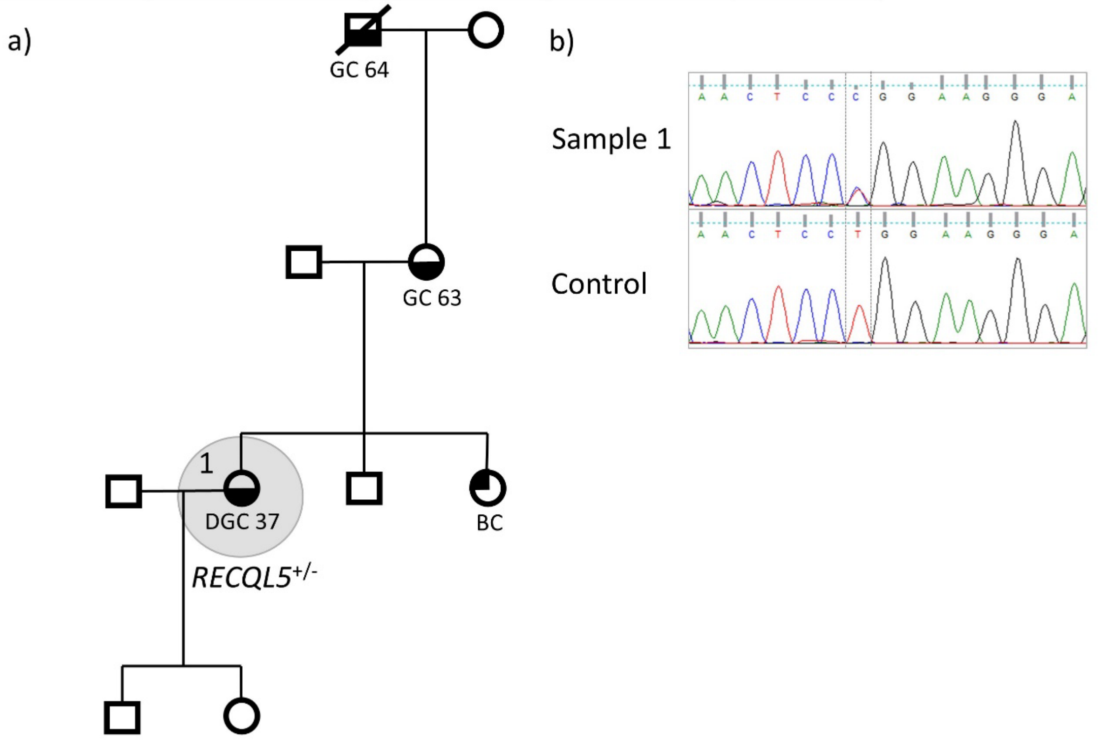
**Supplementary Figure 5:** Family 8 tumour analysis showing representative photomircographs (scale bar 0.2mm) showing a) hematoxylin and eosin b) normal MSH2 expression in tumours. Microsatellites are comparable across c) tumour-free adjacent tissue d) poorly differentiated gastric tissue.

| Gene | Variant | Consequence | Protein Change | Sample 1 | Sample 2 |
|---|---|---|---|---|---|
| *RECQL5* | c.2828C>T | Missense variant | p.Arg943His | C/T | C/C |



**Supplementary Figure 6:** a) The pedigree for family 21. b) Chromatograms showing the *RECQL5* missense variant in the proband against control DNA. Whole exome sequencing was performed on the circled sample, where shading indicates an affected and white indicates an unaffected individual.

| Gene | Variant | Consequence | Protein Change | Sample 1 |
|---|---|---|---|---|
| *RECQL5* | c.2806-2T>C | Splice acceptor variant | | T/C |



**Supplementary Figure 7**: a) The pedigree for family 6. b) Chromatograms showing the *RECQL5* splice acceptor variant in the proband against control DNA. Whole exome sequencing was performed on the circled sample, where shading indicates an affected individual.