InSiGHT Variant Interpretation Committee: Mismatch Repair Gene Variant Classification Criteria

Rules for Variant Classification:

Rules describing the 5 class system for classification of MMR gene variants were devised and documented by Amanda Spurdle and Bryony Thompson in September 2009 for standardised classification of variants in the Colon Cancer Family Registry database, and revised by Amanda Spurdle, Bryony Thompson, Sean Tavtigian and Marc Greenblatt during April 2011, in collaboration with the InSiGHT Variant Interpretation Committee (VIC), modulated by input from committee members during ongoing VIC meetings. They are based on the following:

- The 5 class system described for quantitative assessment of variant pathogenicity in Plon et al.[1], using a multifactorial likelihood model[2-5] as applied to MMR gene variants[6,7];
- The 5 class system for interpretation of splicing variants and aberrations by Spurdle et al.[8];
- The classification of sequence changes according to standard clinical practice – that is, description of variants generally considered pathogenic (clinically relevant in a genetic counselling setting such that germline variant status is used to inform patient and family management) or non-pathogenic (significant evidence against being a dominant high-risk pathogenic mutation); and
- The documentation of non-quantitative methods that have been used to classify variants in the literature.

For a given class, a variant is required to satisfy all the criteria listed for at least one bullet-point that falls within that class. The symbol "✓" represents an "AND" statement. The footnote # describes the rationale for suggested sample numbers, and the rationale for use of indicative MSI or IHC information. The interpretation of functional assays is assisted by a flowchart developed for this purpose (Figure 1).

These criteria provide a baseline for standardized clinical classification of MMR gene sequence variation that may be linked to patient and family management in the genetic counselling arena according to published guidelines [1]. Use of the InSiGHT database, and associated interpretation relating to pathogenicity, is subject to User discretion and responsibility. Whereas InSiGHT has developed processes to assign pathogenicity utilizing high-level expertise available through its membership, such assignments are subject to change with the availability of new information and interpretation processes. Information submitted to the InSiGHT database is available for individual enquiry for clinical use, but any collective use of the data for research or other purposes transgresses agreements InSiGHT has with the valuable community of researchers, clinicians and diagnostic laboratories that generously support the database through their submissions. All users of the database are encouraged to submit their own variants to support the InSiGHT international collaboration to share gene variant data relating to gastrointestinal tumours. Submissions should be directed to the InSiGHT curator John-Paul Plazzer (johnpaul@variome.org).

[a] A known functional protein domain is reported to harbor sequence variants that introduce deleterious changes to protein function (via missense alteration, protein sequence deletion, or protein truncation in the last exon) AND are associated with high risk of cancer. Physical boundaries for functional domains are shown in Figure 2.

[b] As per CMMRD consortium guidelines[11].

[c] Likelihood ratios for segregation can be derived by Bayes factor analysis adapted from the method of Thompson et al[44], as described previously[6]. Penetrance estimates for *MLH1* and *MSH2* variants to be derived from Dowty et al[45] and those for *MSH6* and *PMS2* variants to be derived from Baglietto et al[46] and Senter et al[47].

[d] Standard MSI markers panel: BAT25, BAT26, BAT40, BAT34, D5S346, D17S250, ACTC, D18S55, D10S197, MYCL [45]; D2S123, D18S69 [46]; NR21, NR24, NR27 [47]

[e] Outbred control reference groups currently used for this purpose include datasets from the Exome Aggregation Consortium (exac.broadinstitute.org) and Genome Aggregation Database (gnomad.broadinstitute.org).

[f] Lynch syndrome tumours include: colorectal/colon/rectal, endometrial, ovarian, small bowel/small intestine, renal pelvis, ureter, and stomach/gastric carcinomas, sebaceous skin tumours (adenomas and carcinomas), gliomas.

Important Notes

PMS2 NGS results need confirmation by other orthogonal assays as well as functional assessment (e.g. Long-Range or cDNA), if variants are located in the pseudogene regions (exons 11-15)

Gene-specific penetrance estimates are available at http://lscarisk.org/

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Class 5 – Pathogenic

- Variants with probability of pathogenicity >0.99 (not based on co-segregation in one family, and without conflicting tumour data) using a multifactorial likelihood model
- Variants in the initiation codon of MLH1
- Coding sequence variation resulting in a stop codon i.e. a nonsense or frameshift alteration that is not in the last exon of MSH2, MSH6 or PMS2, and is not after codon 743 in MLH1[a]. For variants in the last exon of MSH2, MSH6 or PMS2 or after codon 743 in MLH1, use other criteria to classify.
- Variants where mRNA assays using **RNA derived from patient constitutional** biological samples indicate that the variant allele results in a splicing aberration (with evidence that the variant allele produces no full-length/reference transcript) leading to premature stop codon or in-frame deletion disrupting a functional domain or protein conformation. **Splicing aberration must be confirmed in a minigene assay or an additional RNA assay from an independent laboratory if it is not a predicted splice site mutation.**
- De novo where both maternity and paternity is confirmed in a case with MMR deficient (MSI/immunoloss consistent with affected gene, with no MLH1 methylation) Lynch spectrum tumour
- Large genomic deletions
- Large genomic duplications shown by laboratory studies (which define the breakpoints of the duplication) to result in a frameshift before the last splice junction
- Variants demonstrating all of the following characteristics with no conflicting results (combined evidence achieves estimated LR >100:1, posterior >0.99 with prior 0.5):

✓ Variant-specific abrogated function in protein or mRNA based lab assays (see MMR functional assay supplementary material, Figure 1)
  - OR - co-occurrence *in trans* with a known pathogenic sequence variant in the same gene in a patient with clinical features consistent with Constitutional Mismatch Repair Deficiency (CMMRD) and documented MMR deficiency in normal cells [b]
  - OR - presence of the variant on different haplotypes across families indicating that reported LS clinical features are not due to an undiscovered sequence change *in cis* with the variant

✓ Evidence for co-segregation with disease where pedigree information provided allows calculation of likelihood ratio (LR) of ≥10:1 [c] or >5 informative meioses
  - OR - at least one revised Amsterdam criteria[9] family with ≥4 affected carriers
  - OR - across ≥2 revised Amsterdam criteria families reported to show segregation with disease (no further information provided)
  - OR - ≥2 families with ≥3 affected non-proband carriers in each family

✓ ≥2 independent MSI-H tumours using a standard panel of 5-10 markers [d] **and/or** loss of MMR protein expression consistent with the variant location (may include tumour information from proband)

✓ evidence that variant is not an undescribed polymorphism at allele frequency >1% (minor allele frequency [MAF] >0.01) in appropriate population control reference groups [e]

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Class 4 – Likely pathogenic

- Variants with probability of pathogenicity between 0.95-0.99 (or >0.99 if co-segregation in one family and presence of conflicting tumour data) using a multifactorial likelihood model
- Variants in the initiation codon of MSH6 or PMS2
- Variants with monoallelic expression - complete loss of expression of the variant allele (full-length transcript should be analysed with and without NMD block)
- De novo with maternity and/or paternity unconfirmed in a case with MMR deficient (MSI/immunoloss consistent with affected gene, with no MLH1 methylation) Lynch spectrum tumour
- Variants at IVS±1 or IVS±2, or G>non-G at last base of exon if first 6 bases of the intron are not GTRRGT, that are untested for splicing aberrations *in vitro*
- Variants demonstrating (combined evidence achieves LR >20:1, posterior 0.95-0.99 with prior 0.5):

✓ variant-specific abrogated function in protein or mRNA based lab assays (see MMR functional assay supplementary material)
  - OR - co-occurrence *in trans* with a known pathogenic sequence variant in the same gene in a patient with clinical features consistent with CMMRD and documented MMR deficiency in normal cells [b]
  - OR - presence of the variant on different haplotypes across families indicating that reported LS clinical features are not due to an undiscovered sequence change *in cis* with the variant

✓ **plus one of the following:**
  - co-segregation with disease assessed by available pedigree information allows calculation of LR of >5:1 [c] or >3 informative meioses
  - OR - at least one revised Amsterdam criteria family with ≥3 affected carriers
  - OR - ≥2 families with ≥2 affected non-proband carriers
  - OR - ≥2 independent tumours with MSI using a standard panel of 5-10 markers [d] **and/or** loss of MMR protein expression consistent with the variant location (may include tumour information from proband)

- A missense substitution that encodes the same amino acid change with a different underlying nucleotide change as a previously established Class 5 pathogenic missense variant with normal RNA result*, and is absent from appropriate population control reference groups [e]
  * Otherwise, if the previously established Class 5 pathogenic missense variant truly is a splice defect, the new missense variant also has to be investigated on a functional level for RNA splicing.

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[c] Likelihood ratios for segregation can be derived by Bayes factor analysis adapted from the method of Thompson et al[44], as described previously[6]. Penetrance estimates for *MLH1* and *MSH2* variants to be derived from Dowty et al[45] and those for *MSH6* and *PMS2* variants to be derived from Baglietto et al[46] and Senter et al[47].
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Class 3 – Uncertain

- Variants with probability of pathogenicity between 0.05-0.949 using a multifactorial likelihood model
- Variants in the initiation codon of MSH2
- Variants that have insufficient evidence (molecular or otherwise) to classify, which may include large genomic duplications not yet shown by laboratory studies to result in a frameshift before the last splice junction, missense alterations, small in-frame insertions/deletions, silent variants, intronic variants, promoter and regulatory region variants

Conflicting lines of evidence (e.g. IHC/MSI does not fit, segregation does not fit, etc)

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Class 2 – Likely not pathogenic/little clinical significance

- Variants with probability of pathogenicity between 0.001-0.049 using a multifactorial likelihood model
- Synonymous substitutions and intronic variants with no associated mRNA aberration (either splicing or allelic imbalance) as determined by laboratory assays conducted with nonsense-mediated decay inhibition. Whenever abnormal transcripts are identified at similar levels in controls they will be considered naturally occurring isoforms and not mRNA aberrations
- Variants reported to occur in a specific ethnic group at allele frequency ≥1%, (MAF ≥ 0.01, tested in ≥ 160 individuals) and that have not yet been excluded as known founder pathogenic sequence variants ("founder mutations")
- 5' UTR or promoter region variants which have bi-allelic expression, shown by cDNA experiment that both alleles are expressed ~ 50/50 including one with the variant or at least one heterozygous exonic variant in the transcript (full-length transcript should be analysed with and without NMD block)
- Variants demonstrating:

✓ variant-specific proficient function in protein and mRNA based lab assays (see MMR functional assay supplementary material)
- OR - co-occurrence *in trans* with a known pathogenic sequence variant in the same gene in a patient with colorectal cancer after age 45 (or other LS cancer above the median age of onset for that cancer in LS [f]), and who has no previous or current evidence of clinical manifestations of CMMRD

✓ **plus one of the following:**
- present in control reference groups at allele frequency 0.01-1% (MAF 0.0001-0.01, tested in ≥ 160 individuals)
- OR - lack of co-segregation with disease consistent with a dominant high-risk pathogenic sequence variant in pedigrees (LR≤ 0.01 [c])
- OR - estimated risk with upper bound 95% confidence limit <4, as determined by large well-designed case-control studies that consider size, geography/ethnicity and quality control measures
- OR - ≥3 colorectal tumours with MSS **and/or** no loss of MMR protein expression **and/or** loss of MMR protein(s) in LS spectrum tumours, that is inconsistent with the gene demonstrating genetic variation

- Variants demonstrating any **three** of the following:
- present in control reference groups at allele frequency 0.01-1% (MAF 0.0001-0.01, tested in ≥ 160 individuals)
- OR - lack of co-segregation with disease consistent with a dominant high-risk pathogenic sequence variant in pedigrees (LR≤ 0.01 [c])
- OR - estimated risk with upper bound 95% confidence limit <4, as determined by large well-designed case-control studies that consider size, geography/ethnicity and quality control measures

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[e] Outbred control reference groups currently used for this purpose include datasets from the Exome Aggregation Consortium (exac.broadinstitute.org) and Genome Aggregation Database (gnomad.broadinstitute.org).
[f] Lynch syndrome tumours include: colorectal/colon/rectal, endometrial, ovarian, small bowel/small intestine, renal pelvis, ureter, and stomach/gastric carcinomas, sebaceous skin tumours (adenomas and carcinomas), gliomas.

- OR - ≥3 colorectal tumours with MSS **and/or** no loss of MMR protein expression **and/or** loss of MMR protein(s) in LS spectrum tumours, that is inconsistent with the gene demonstrating genetic variation

- An exonic variant, that encodes the same amino acid change as a previously established Class 1 missense variant with a different underlying nucleotide change, and for which there is no evidence of mRNA aberration (splicing or allelic imbalance) as determined using *in vitro* laboratory assays conducted with nonsense-mediated decay inhibition

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Class 1 – Not pathogenic/no clinical significance

- Variants with probability of pathogenicity <0.001 using a multifactorial likelihood model
- Variants reported to occur in control reference groups at allele frequency ≥1% (MAF ≥ 0.01, tested in ≥ 160 individuals) and excluded as founder pathogenic sequence variants
- 5' UTR or promoter region variants demonstrating:

✓ Bi-allelic expression, shown by cDNA experiment that both alleles are expressed ~ 50/50 including one with the variant or at least one heterozygous exonic variant in the transcript (full-length transcript should be analysed with and without NMD block)

✓ **Meets Class 1 Frequency criteria:**
present in control reference groups at allele frequency ≥ 0.01% (MAF ≥ 0.0001, tested in ≥ 160 individuals)

- OR - lack of co-segregation with disease consistent with a dominant high-risk pathogenic sequence variant in pedigrees (LR≤ 0.01 [c])

- Variants demonstrating:

✓ Variant-specific proficient function in protein and mRNA based lab assays (see MMR functional assay supplementary material)
- OR - co-occurrence *in trans* with a known pathogenic sequence variant in the same gene in a patient with colorectal cancer after age 45 (or other LS cancer above the median age of onset for that cancer in LS [f]), and who has no previous or current evidence of clinical manifestations of CMMRD

✓ **Plus any two of the following:**
- present in control reference groups at allele frequency 0.01-1% (MAF 0.0001-0.01, tested in ≥ 160 individuals)
- **OR -** lack of co-segregation with disease consistent with a dominant high-risk pathogenic sequence variant in pedigrees (LR≤ 0.01 [c])
- **OR -** estimated risk with upper bound 95% confidence limit <4, as determined by large well-designed case-control studies that consider size, geography/ethnicity and quality control measures
- **OR -** ≥3 colorectal tumours with MSS **and/or** no loss of MMR protein expression **and/or** loss of MMR protein(s) in LS spectrum tumours, that is inconsistent with the gene demonstrating genetic variation

- Variants demonstrating all of the following:

✓ Present in control reference groups at allele frequency 0.01-1% (MAF 0.0001-0.01, tested in ≥ 160 individuals)

✓ Lack of co-segregation with disease consistent with a dominant high-risk pathogenic sequence variant in pedigrees (LR≤ 0.01 [c])

✓ Estimated risk <1.5, with upper bound 95% confidence limit <4, as determined by large well-designed case-control studies that consider size, geography/ethnicity and quality control measures

✓ ≥3 colorectal tumours with MSS **and/or** no loss of MMR protein expression **and/or** loss of MMR protein(s) in LS spectrum tumours, that is inconsistent with the gene demonstrating genetic variation

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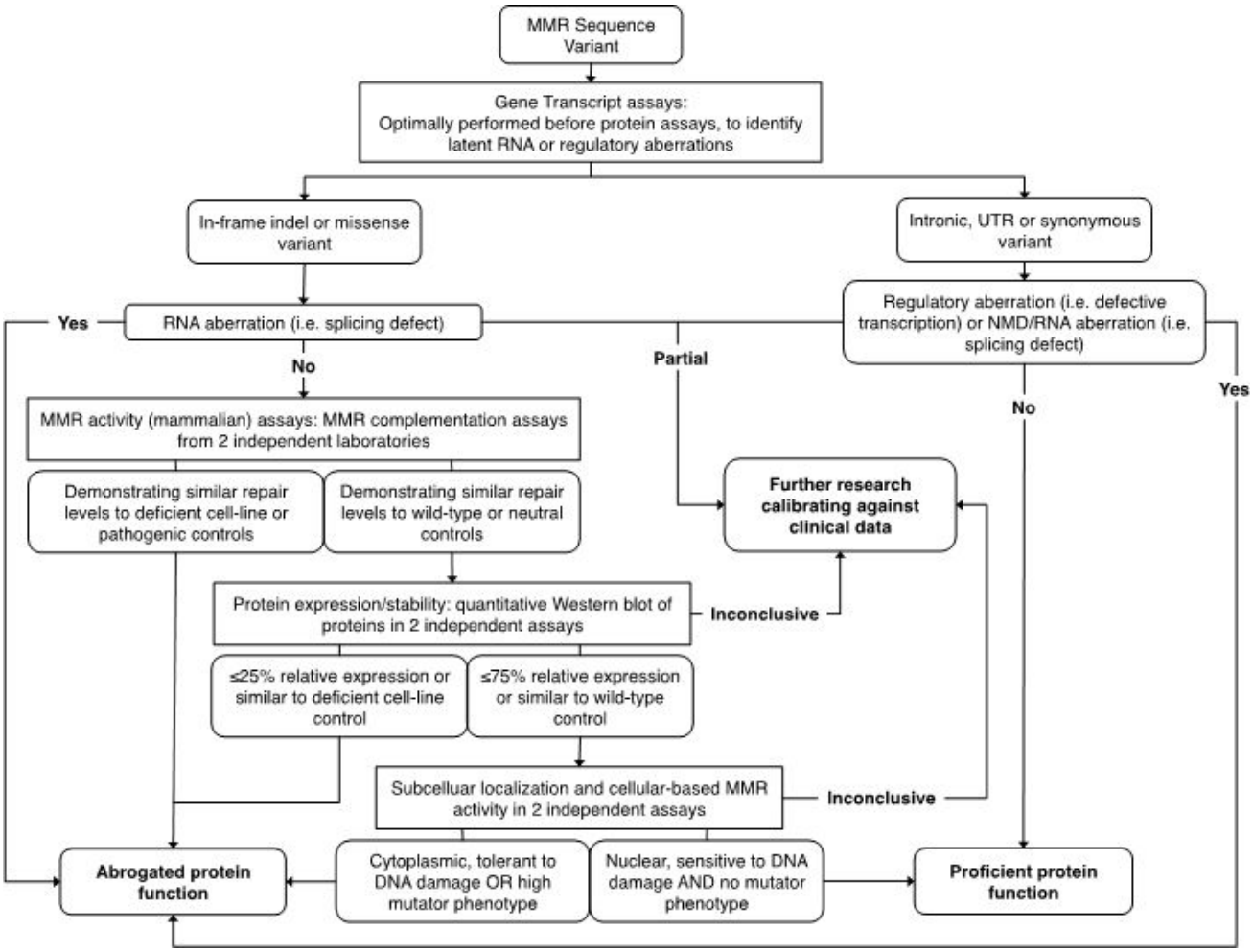

Figure 1. Flowchart used to assist in interpretation of available functional assay data. For variants that had normal/inconclusive/intermediate MMR activity in 2 independent assays, but deficient protein function in 2 independent assays, abrogated function was assigned. NMD – nonsense mediated decay.

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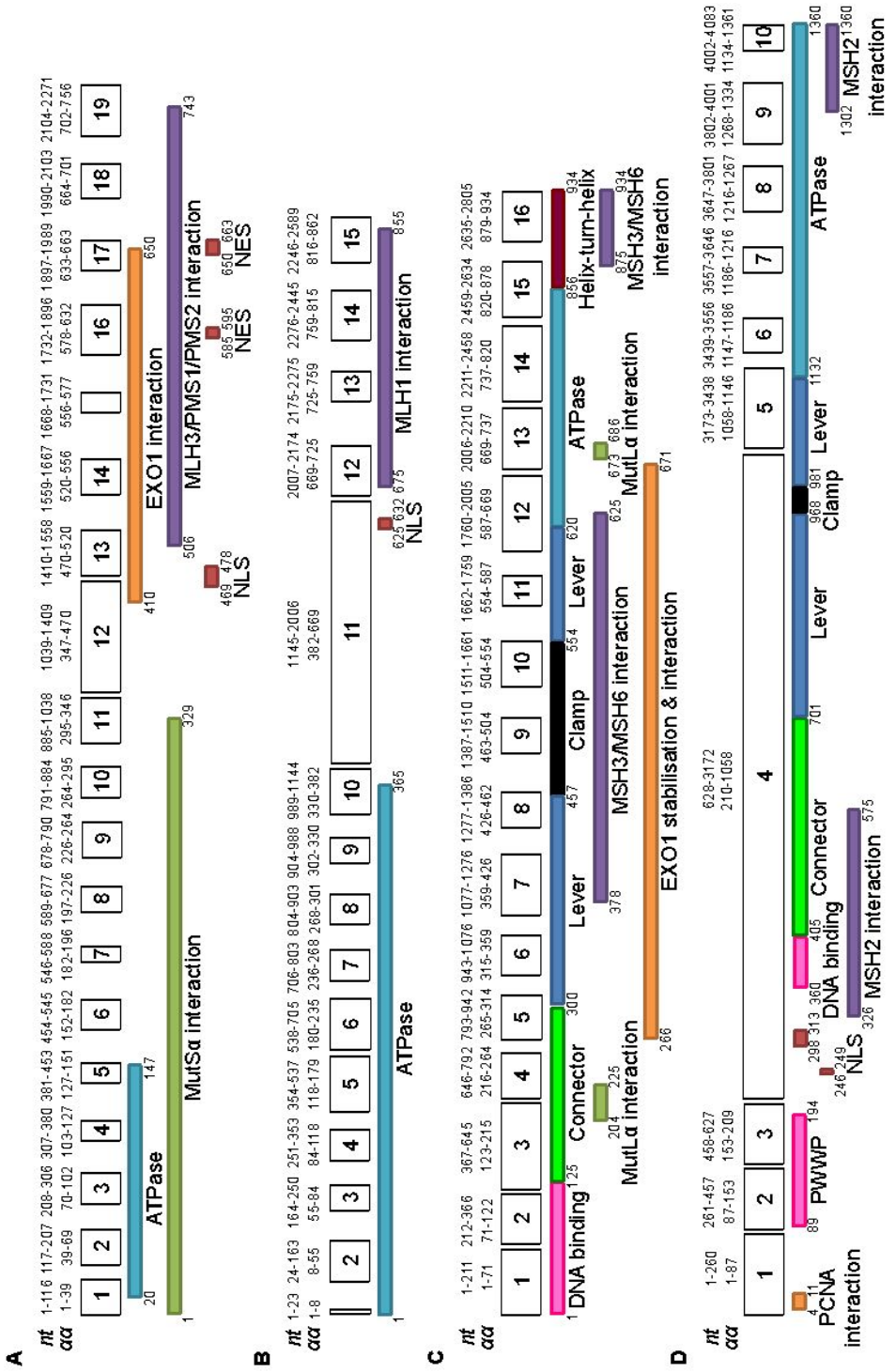

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Figure 2. Linear schematic of mismatch repair gene structure and functional domains. The nucleotide (nt) and amino acid (αα) positions are numbered for the exon boundaries of *MLH1* (A), *PMS2* (B), *MSH2* (C), and *MSH6* (D). Position numbering is based on Human Genome Variation Society (HGVS) nomenclature[12]. The proteins include the following functional domains: ATPase domain – MLH1[13-18], PMS2[19], MSH2[20,21], and MSH6[21-23]; MutSα interaction in MLH1[17,24]; EXO1 interaction and/or stabilisation domain – MLH1 and MSH2[25]; nuclear localisation signal (NLS) and/or nuclear export signal (NES) – MLH1[26], PMS2[27], and MSH6[28]; MLH1/MLH3/PMS1/PMS2 interaction domain – MLH1 and PMS2[29,30]; DNA binding domain – MSH2[20,31] and MSH6[32]; connector and lever domains – MSH2[20,31] and MSH6[22]; MutLα interaction domains in MSH2[33]; clamp domain – MSH2[20,31] and MSH6[34]; helix-turn-helix domain in MSH2[20,31]; MSH2/MSH3/MSH6 interaction domains – MSH2 and MSH6[35]; PCNA interaction domain in MSH6[36]; PWWP domain in MSH6 binds double-stranded DNA non-specifically[37].

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Footnotes to Mismatch Repair Gene Variant Classification Guidelines:

The upper 95% confidence limit of frequency for an allele observed once in 160 individuals (320 chromosomes) is <0.01, the allele frequency considered sufficient for classification as class 1 (not pathogenic/low clinical significance). Characteristics of a pathogenic variant were selected to achieve a combined likelihood ratio of >100:1 in conjunction with a prior of 0.5 (estimated prior irrespective of *in silico* predictions). Namely, the co-segregation and family history descriptions were selected to estimate segregation odds of 10:1, results from functional assays were assumed to have likelihood ratio 5:1 and MSI/IHC data was conservatively assumed to carry a likelihood ratio of 5:1. Tumour data was based on two tumours for MSI and/**or** IHC to allow for the possibility that pathogenic missense alterations may not all demonstrate loss of MMR protein expression. Where both MSI and IHC information are available, MSI-H results will take precedence over normal immunohistochemical results, since MSI is more specific than MMR immunohistochemistry in colorectal cancers[38,39]. For variants that demonstrate tumour MSS and loss of MMR protein expression, technical or other explanations[38,40-42] for the discrepancy should be investigated. The need for multiple results was implemented to minimize the chance of a "sporadic" MSI-H or negative immunohistochemical result for MLH1 methylated tumours (0.15x0.15 = 0.0225). Independent tumours can include multiple primary tumours from a single individual.

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