

Novel *GPR34* and *CCR6* mutation and distinct genetic profiles in MALT lymphomas of different sites

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ABSTRACT

Mucosa-associated lymphoid tissue (MALT) lymphoma originates from a background of diverse chronic inflammatory disorders at various anatomic sites. The genetics underlying its development, particularly in those associated with autoimmune disorders, is poorly characterized. By whole exome sequencing of 21 cases of MALT lymphomas of the salivary gland and thyroid, we have identified recurrent somatic mutations in 2 G-protein coupled receptors (*GPR34* and *CCR6*) not previously reported in human malignancies, 3 genes (*PIK3CD*, *TET2*, *TNFRSF14*) not previously implicated in MALT lymphoma, and a further 2 genes (*TBL1XR1*, *NOTCH1*) recently described in MALT lymphoma. The majority of mutations in *GPR34* and *CCR6* were nonsense and frameshift changes clustered in the C-terminal cytoplasmic tail, and would result in truncated proteins that lack the phosphorylation motif important for β -arrestin-mediated receptor desensitization and internalization. Screening of these newly identified mutations, together with previously defined genetic changes, revealed distinct mutation profiles in MALT lymphoma of various sites, with those of salivary gland characterized by frequent *TBL1XR1* and *GPR34* mutations, thyroid by frequent *TET2*, *TNFRSF14* and *PIK3CD* mutations, and ocular adnexa by frequent *TNFAIP3* mutation. Interestingly, in MALT lymphoma of the salivary gland, there was a significant positive association between *TBL1XR1* mutation and *GPR34* mutation/translocation ($P=0.0002$). In those of ocular adnexa, *TBL1XR1* mutation was mutually exclusive from *TNFAIP3* mutation ($P=0.049$), but significantly associated with IGHV3-23 usage ($P=0.03$) and *PIK3CD* mutation ($P=0.009$). These findings unravel novel insights into the molecular mechanisms of MALT lymphoma and provide further evidence for potential oncogenic co-operation between receptor signaling and genetic changes.

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Introduction

Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) originates from acquired MALT at diverse anatomic sites. Accordingly, its development is closely associated with distinct inflammatory disorders. For example, MALT lymphomas of the stomach and ocular adnexa are associated with chronic infection by *Helicobacter pylori* and *Chlamydia psittaci*, respectively, while those of the salivary gland and thyroid are associated with Sjögren syndrome and Hashimoto thyroiditis, respectively.¹ In addition to its diverse etiology, MALT lymphomas of various sites also show distinct biased usage of IGHV. For instance, up to one-third of ocular adnexal MALT lymphomas have IGHV4-34 BCR that is most likely auto-reactive, binding to the carbohydrate I/i antigens, while more than 50% of salivary gland MALT lymphomas bear IGHV1-69 BCR with features of rheumatoid factors.^{2,9} These findings suggest that various chronic inflammatory disorders may generate immune responses, which preferentially expand B cells with certain properties, such as autoreactive BCR, consequently leading to the development of MALT lymphoma.^{1,10}

There are a number of genetic changes described in MALT lymphomas, but many of these changes occur at considerable variable frequencies at different sites despite the fact that they commonly affect the NF- κ B pathway.¹ For example, t(11;18)(q21;q21)/API2-MALT1 and to a lesser extent t(1;14)(p22;q32)/BCL10-IGH, are frequently seen in MALT lymphoma of the lung and stomach, but are rarely or not seen in those of the salivary gland and thyroid.¹¹⁻¹³ In contrast, *TNFAIP3* mutation and/or deletion, which encodes a NF- κ B negative regulator, is frequent in MALT lymphoma of the ocular adnexa, but occurs at low frequencies in those of other sites.¹⁴⁻¹⁶ The reason for such dramatic differences in the genetics of MALT lymphomas of different sites is unclear, and it remains to be investigated whether the occurrence of these genetic changes is influenced by the distinct background disorders or *vice versa*.

The development of MALT lymphoma is the result of oncogenic co-operation between immunological drive and acquired genetic changes as neither the above genetic changes nor the immunological drive alone is sufficient for malignant transformation. Nevertheless, the exact mechanisms of oncogenic co-operation in MALT lymphoma of various sites remain largely elusive. In a recent study, we found a significant association between *TNFAIP3* inactivation and biased IGHV4-34 usage in ocular adnexal MALT lymphoma, arguing for their co-operation in sustaining chronic BCR signaling, and thus NF- κ B activation.¹⁷ Interestingly, a very similar finding, namely a significant association between biased usage of IGHV1-2 and inactivating mutation of *KLF2* (a negative regulator of NF- κ B), was previously reported in splenic marginal zone lymphoma.¹⁸ Taken together, such co-operation between antigenic stimulation and genetic abnormalities may represent a common feature in marginal zone B-cell lymphoma. Nonetheless, this has not been fully investigated in marginal zone lymphoma of various sites, particularly in those of the salivary gland and thyroid, where very few genetic features have been identified. In the present study, we first investigated the mutation profile of 21 MALT lymphomas of the salivary gland and thyroid by whole exome sequencing (WES), then validated the recurrent mutations in MALT lymphoma of various sites by targeted sequencing, and finally investigated their association with IGHV usage.

Methods

Patients' samples and DNA extraction

A total of 249 cases of MALT lymphoma were included in this study, originating from the ocular adnexa (n=115), salivary gland (n=58), stomach (n=36), thyroid (n=13), lung (n=13), and other sites (n=14); 179 of these cases had been the subject of a previous study for somatic mutation in 17 genes.¹⁷ Genomic DNA was extracted from tumor rich areas (>40%) of formalin fixed paraffin embedded (FFPE) lymphoma biopsies in 217 cases, and where possible from non-neoplastic cells by microdissection using the QIAamp DNA micro kit (Qiagen, the Netherlands). Additionally, high molecular weight DNA was available in 32 cases of MALT lymphoma. DNA quality was assessed by PCR amplification of variably sized genomic fragments, with those amenable to PCR of ≥ 300 bp genomic fragment used for mutation analyses by targeted sequencing.¹⁹ Among the cases included in this study, clinical information such as site involvement and treatment details were available in 98 cases of ocular adnexal MALT lymphomas, and their correlation with genetic changes was examined. Local ethical guidelines for the use of archival tissues for research were adopted with the approval of the ethics committees of the institutions involved.

Whole exome sequencing

A total of 21 MALT lymphomas (14 from salivary gland, 7 from thyroid) together with one matched non-neoplastic biopsy were investigated by whole exome sequencing (WES) (*Online Supplementary Table S1*). The initial 2 cases (one thyroid and one salivary gland MALT lymphoma) were carried out on high molecular weight tumor DNA at the Eastern Sequence and Informatics Hub (EASIH). Genomic libraries were prepared using the Illumina Truseq DNA sample preparation v.2 kit (Illumina, CA, USA), captured with the Nimblegen SeqCap EZ Exome v.3 (Roche, Germany) and sequenced on an Illumina HiSeq platform. The remaining 19 cases (6 thyroid and 13 salivary gland MALT lymphoma) together with one matched non-neoplastic sample were performed on FFPE tissue DNA at the Wellcome Trust Sanger Institute. Genomic libraries were generated using the Illumina Paired End Sample Prep Kit, enriched using the Agilent SureSelect Human All Exon 50Mb kit (Agilent, CA, USA), and sequenced on an Illumina HiSeq platform using a 75bp paired end protocol. Sequencing reads were aligned to the hg19 reference genome using BWA with default settings, with single nucleotide variants called by CaVEMan (Cancer Variants through Expectation Maximization), and insertions and deletions by PINDEL. Data were filtered against 300 unmatched normal controls available from the Cancer Genome Project in addition to the 1000 genomes project and Ensembl variation databases to remove known single nucleotide polymorphisms (SNP).

Somatic variant validation by fluidigm access array PCR and illumina MiSeq sequencing

Where indicated, the novel and shortlisted variants identified by WES were confirmed by PCR and Sanger Sequencing, and their somatic origin ascertained by PCR and Sanger sequencing analysis of DNA samples from microdissected non-neoplastic cells.

Mutations in *CCR6*, *FGFR3*, *FOXO1*, *GPR34*, *IKBKB*, *PIK3CD*, *NOTCH1*, *TBL1XR1*, *TET2* and *TNFRSF14* were then screened using the Fluidigm Access Array PCR and Illumina MiSeq protocol, as previously described.¹⁹ Primer sequences and PCR conditions are shown in *Online Supplementary Table S2*. In addition, mutation in a further 17 genes including BCR signaling (*CD79A*, *CD79B*, *CARD11*), NF- κ B (*TNFRSF11A*, *TNFAIP3*, *TRAF3*), TLR signaling (*MYD88*), plasma cell differentiation (*PRDM1*), histone modifiers

(*CREBBP*, *EP300*, *EZH2*, *MLL2*, *MEF2B*, *KDM2B*), antigen presentation (*B2M*, *CD58*), and apoptosis (*TP53*) were similarly screened in the cases (n=70) that were not investigated previously.¹⁷ Briefly, DNA samples were sequenced in duplicate and analyzed using an in-house variant calling protocol optimized against a large panel of known mutations.¹⁹ Variants that appeared in both replicates at $\geq 10\%$ AAF (alternative allele frequency) were further validated by inspection of their bam files, and then considered as true genetic changes.¹⁹ Where possible, variants were confirmed as somatic by PCR and Sanger sequencing of non-neoplastic DNA or assumed to be somatic if previously reported as somatic in the COSMIC database or other samples in the cohort.

Fluorescence *in situ* hybridization analysis

Fluorescence *in situ* hybridization (FISH) analysis was performed on FFPE tissue sections with Vysis LSI IGH Dual Colour Break Apart Probe (Abbott Molecular, IL, USA) and an in house GPR34 Dual Colour Break Apart probe, as previously described.^{16,20} To generate the in-house GPR34 break-apart probe, BAC clones RP11-643E21 and RP11-524P6 centromeric to *GPR34*, and RP11-360E17 and CTD-3202J9 telomeric to *GPR34* were amplified using the Templphi kit (GE Healthcare, IL, USA) and then labeled with SpectrumOrange and SpectrumGreen respectively using nick translation with random priming (Abbott Molecular, IL, USA).^{16,20}

Sequencing analysis of the rearranged immunoglobulin heavy chain genes

The sequence of clonally rearranged immunoglobulin heavy chain genes was available in 94 cases from a previous study.¹⁷ Additional PCR and sequencing of the rearranged immunoglobulin heavy chain genes were performed for the cases investigated by WES using the BIOMED-2 VH FR1-JH or FR2-JH primers, as described previously.¹⁷ Sequences were analyzed using IMGIT/V-Quest software (www.imgt.org/IMGIT_vquest/vquest), and successfully annotated in a total of 101 cases (*Online Supplementary Table S3*).

Statistical analysis

Fishers exact test was used to test for associations between categorical variables.

Results

Novel mutations in MALT lymphomas identified by WES

Whole exome sequencing was successful in a total of 21 MALT lymphomas (14 from salivary gland, 7 from thyroid) together with one matched non-neoplastic DNA (*Online Supplementary Table S4*). After filtering known SNPs, synonymous changes and benign variants by Polyphen2, 72 variants were seen in the case with matched germline DNA, while an average of 111 variants (range 46-264/case) were observed in the remaining cases without matched germline DNA (*Online Supplementary Figure S4*). Based on their frequencies, potential functional impact, involvement in cancer and lymphocyte biology and hits against relevant GO terms, novel variants in 10 genes were selected for validation and their somatic origin was confirmed by Sanger sequencing of corresponding non-neoplastic DNA (*Online Supplementary Table S5*). These shortlisted genes included 2 G protein-coupled receptors (*GPR34*, *CCR6*) not yet reported as a mutation target in human malignancies, 6 (*FGFR3*, *FOXO1*, *IKBK*, *PIK3CD*, *TET2*, *TNFRSF14*) not yet implicated in MALT lymphoma, and 2 (*TBL1XR1*, *NOTCH1*) recently described in MALT lymphoma (*Online*

Supplementary Table S4 and *Online Supplementary Figure S2*).²¹ All of these mutations identified by WES were potentially pathogenic (see section below). To further investigate their mutation frequency and characteristics in MALT lymphoma, we screened a large cohort from various anatomic sites for mutations by Fluidigm Access Array PCR and Illumina MiSeq sequencing.

Variable involvement of newly identified mutations in MALT lymphoma of different sites

A total of 249 cases of MALT lymphoma from the ocular adnexa (n=115), salivary gland (n=58), stomach (n=36), thyroid (n=13), lung (n=13), and other sites (n=14) were investigated for somatic mutations in 27 genes with the 10 genes mentioned above investigated exclusively by the present study, and 17 genes studied by previous (n=149) and present (n=70) studies, respectively (*Online Supplementary Tables S6* and *S7*).¹⁷ Recurrent genetic changes are shown in Figure 1 (*Online Supplementary Figure S3*). Interestingly, the findings showed remarkable variations in their involvement among MALT lymphoma of different sites.

GPR34 mutation was almost exclusively found in MALT lymphoma of salivary gland (9 of 56, 16%), with the exception of a single case from ocular adnexa (Figure 1). The majority of *GPR34* mutations were nonsense changes (n=6) and frameshift indel (n=1) that were clustered in the C-terminal regulatory regions, resulting in truncated proteins (Figure 2). The remaining 3 mutations were missense changes, namely R84H, D151A and Y327N.

GPR34 is a G-protein coupled receptor (GPCR) and is deregulated by t(X;14)(p11.4;q32) in MALT lymphoma of salivary gland and lung with a history of Sjögren syndrome.^{20,22,23} In view of this finding, we screened salivary gland MALT lymphoma for t(X;14)(p11;q32) by interphase FISH, and identified the translocation in 2 of 58 (3%) cases (*Online Supplementary Figure S4*). Interestingly, *GPR34* mutation and translocation were mutually exclusive, together seen in 11 of 58 (19%) cases of salivary gland MALT lymphoma (Figure 1 and *Online Supplementary Figure S4*).

TBL1XR1 mutation was most frequent in MALT lymphoma of salivary gland (14 of 58, 24%), occurring less frequently in MALT lymphomas of the stomach (3 of 36, 8%), lung (1 of 13, 8%), ocular adnexa (7 of 115, 6%), skin (1 of 8, 13%) and in a single case from tonsil, but not in the thyroid (Figure 1). A total of 30 mutations were found in 27 cases, with 3 cases affected by two mutations. The majority of these mutations (20 missense changes and 5 inframe deletions) were within the WD40 domains and frequently affected regions or residues of structural importance, which are likely critical for interaction with NCoR (Figure 3). The remaining 5 mutations included 3 substitution changes at exon 11 splice site, one frameshift deletion and one nonsense change, most likely resulting in truncated proteins with potential increased binding to UbCH5.²⁴

TET2 mutation was found in the majority of MALT lymphomas of the thyroid (8 of 13, 62%), and also recurrently seen in those of salivary gland (5 of 58, 9%), ocular adnexa (5 of 115, 4%), stomach (3 of 3, 8%), and lung (1 of 13, 8%) (Figure 1). A total of 31 *TET2* mutations were detected in 23 cases, with 8 cases showing multiple mutations (Figure 2). Interestingly, 7 of 8 of the cases with multiple *TET2* mutations were MALT lymphomas of the thyroid. The vast majority of these mutations were deleterious changes including 11 frameshift indels, 4 nonsense, one splice site,

and 15 missense mutations. Of these, the majority of frameshift (8 of 11) and nonsense (3 of 4) mutations were found in MALT lymphomas of the thyroid.

To our surprise, *TNFRSF14* mutation was highly frequent in MALT lymphoma of thyroid (6 of 13, 46%), albeit infrequent in those of other sites (Figure 1). A total of 21 *TNFRSF14* mutations were detected in 18 cases, and all mutations were deleterious changes comprising 6 nonsense, 2 frameshift indel, one inframe deletion, 11 missense changes including 2 in the first codon thus abolishing the translation start site, and a single splice site mutation (Figure 2). As *TNFRSF14* mutation is common in pediatric-type follicular lymphoma and the mutations identified are of a similar nature,²⁵ we carefully reviewed the histopathology and immunophenotype of our cases with *TNFRSF14* mutations, where necessary performing further immunohistochemistry for CD10 and BCL6, and also FISH for *BCL2* and *BCL6* translocation. These additional analyses confirmed the original diagnosis of MALT lymphoma in these cases.

PIK3CD was another gene frequently mutated in MALT lymphoma of thyroid (3 of 13, 23%), followed by salivary gland (5 of 58, 9%), but rarely in those of other sites (Figure 1). A total of 11 *PIK3CD* mutations were identified in 11 MALT lymphomas, and the vast majority of these mutations were missense changes including N334K and E1021K, previously reported in patients with activated PI3K δ syndrome (hyper IgM syndrome) and in DLBCL (Figure 2).²⁶⁻²⁹

Mutation in the remaining genes was relevantly infre-

quent, without any apparent bias among the sites examined. Mutation in *CCR6* was recurrent, and seen in a total of 7 MALT lymphomas, with 7 mutations including 5 deleterious changes (3 nonsense, 3 frameshift indels) and 2 missense changes, largely clustered in the C-terminal regulatory region (Figures 1 and 2). Interestingly, mutations in *GPR34* and *CCR6*, both members of the GPCR family, were mutually exclusive.

Distinct association of genetic changes in MALT lymphoma of various sites

Correlation analysis revealed several distinct associations among genetic changes in MALT lymphoma of different sites. In salivary gland MALT lymphoma, there was a significant association between *TBL1XR1* mutation and *GPR34* mutation/translocation ($P=0.0002$), with *TBL1XR1* mutation seen in 8 of 11 (73%) cases with *GPR34* mutation/translocation, but only in 6 of 47 (13%) cases without *GPR34* genetic abnormalities (Figure 4 and *Online Supplementary Figure S5*). Both *GPR34* and *TBL1XR1* genetic changes were more frequently seen in the cases with non-IGHV1-69 than those with IGHV1-69 rearrangement (29% vs. 9%; 57% vs. 18%, respectively) although this does not reach statistical significance.

In ocular adnexal MALT lymphoma, *TBL1XR1* mutation was mutually exclusive from *TNFAIP3* mutation ($P=0.049$), but significantly associated with *PIK3CD* mutation ($P=0.009$), with *TBL1XR1* mutation seen in 2 of 3 (67%)

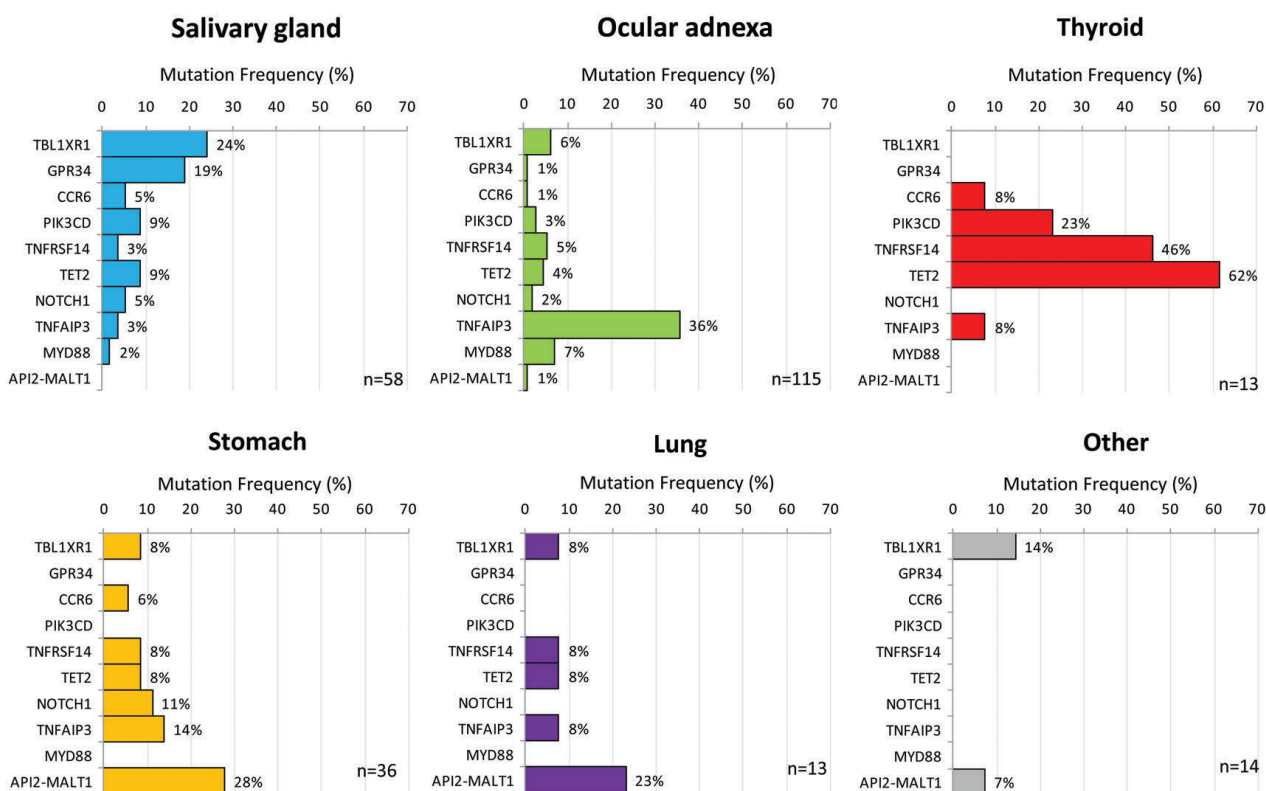


Figure 1. Distinct mutation profiles in mucosa-associated lymphoid tissue (MALT) lymphoma of various sites. A total of 27 genes including the 10 MALT lymphoma-associated genes identified in the present study were screened for mutation by targeted sequencing, but only those showing recurrent changes are presented here. API2-MALT1 denotes t(11;18)(q21;q21) from previous studies.^{11,12} *GPR34* results in MALT lymphoma of the salivary gland include both mutation and t(X;14)(p11;q32)/*GPR34-IGH*. MALT lymphomas of the salivary gland are featured by frequent *TBL1XR1* and *GPR34* mutations, while those of the thyroid by frequent *TET2*, *TNFRSF14* and *PIK3CD* mutations, and ocular adnexa by frequent *TNFAIP3* mutation.

cases with *PIK3CD* mutation, but in only 5 of 112 (4%) cases without *PIK3CD* mutation (Figure 4 and *Online Supplementary Figure S5*). *TBL1XR1* mutation was also mutually exclusive from IGHV4-34 rearrangement, but significantly associated with IGHV3-23 usage ($P=0.03$), with *TBL1XR1* mutation seen in 3 of 11 (27%) cases with IGHV3-23 rearrangement, but in only 2 of 52 (4%) cases with other rearrangements (Figure 4). *MYD88* mutation also appeared to be more frequent in *TBL1XR1* mutated cases (2 of 7, 29%) compared to non-mutated cases (6 of 108, 6%), although this did not reach significance ($P=0.07$).

In gastric MALT lymphoma, t(11;18) API2-MALT1 was mutually exclusive from other genetic changes ($P=0.025$) (*Online Supplementary Figure S5*).

Correlation between genetic changes and clinicopathological parameters

This was carried out in 98 cases of ocular adnexal MALT lymphoma where clinicopathological data were available (*Online Supplementary Table S8*). *TBL1XR1* mutation and IGHV3-23 usage were significantly associated with involvement of the conjunctiva ($P=0.002$ in each, respectively), while *TNFRSF14* mutation was significantly associated with involvement of the orbit ($P=0.04$). *MYD88* and *TP53* mutations were much higher in cases involving both orbit and conjunctiva than those involving only a single site but

only the former showing a statistical significance (4 of 23, 17% vs. 2 of 75, 3%; $P=0.03$). With the exception of *TNFAIP3* deletion/mutation that was significantly associated with higher radiation dosages to achieve complete remission as reported previously,¹⁶ none of the other genetic changes showed any significant association with radiation dosages.

Discussion

By WES of 21 cases of MALT lymphomas of the salivary gland and thyroid, we have identified several novel genetic changes in MALT lymphoma, including *GPR34* and *CCR6* mutations not yet previously reported in human malignancies. Screening these newly identified mutations revealed distinct mutation profiles in MALT lymphoma of various sites, and provides further evidence of potential oncogenic co-operation between receptor signaling and genetic changes.

GPR34 and *CCR6* mutations in MALT lymphoma

Both *GPR34* and *CCR6* are members of class A G protein-coupled receptor (GPCR) superfamily, which transduce extracellular stimulation into intracellular signaling through G protein and β -arrestin. By interacting with G protein,

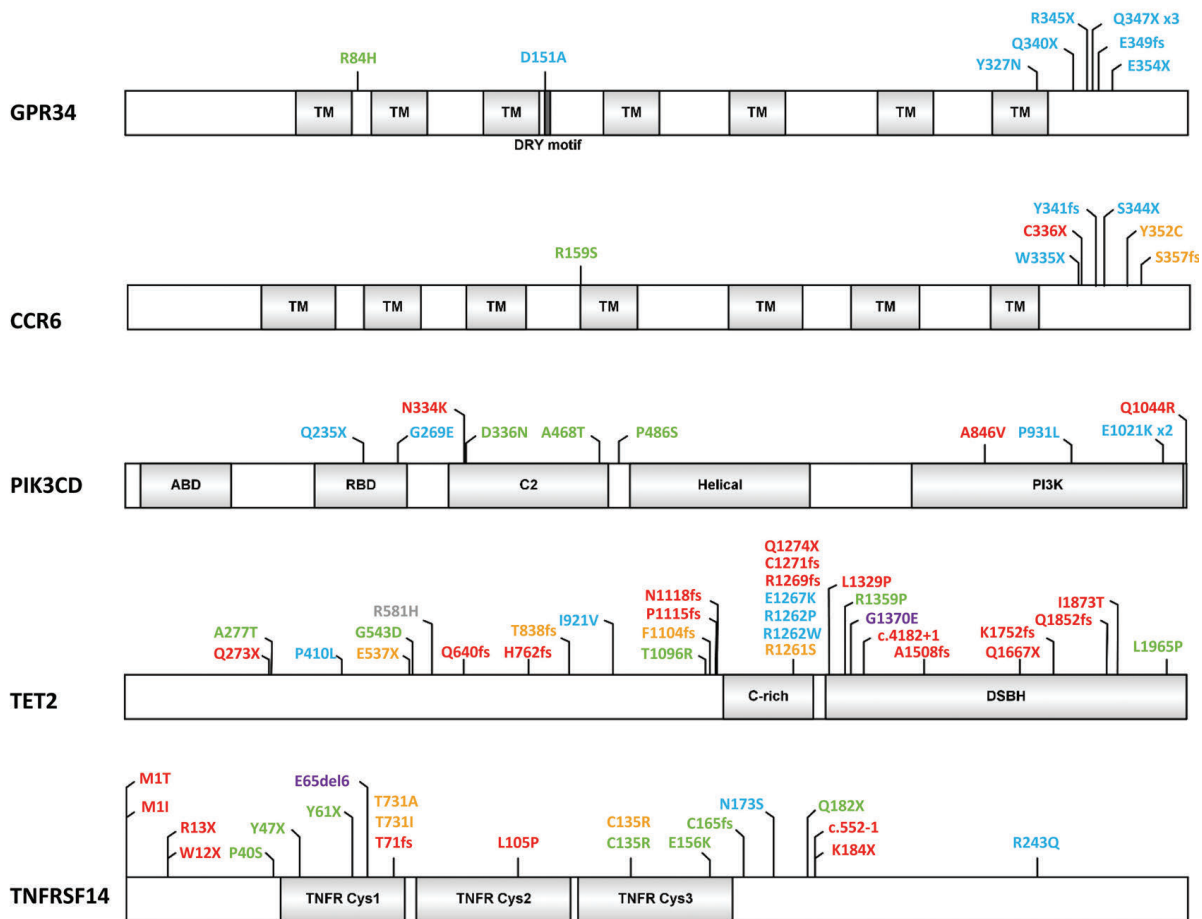


Figure 2. Nature and distribution of mutations in newly identified mucosa-associated lymphoid tissue (MALT) lymphoma-associated genes. Site of MALT lymphoma in which the mutation was identified is indicated by color (blue: salivary gland; red: thyroid; green: ocular adnexa; orange: stomach; purple: lung; gray: other sites). TM: transmembrane domain; ABD: adaptor binding domain; RBD: ras binding domain; DSBH: double-stranded β -helix.

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