

In-Silico Identification and Structural Characterization of a Recurrent KRAS Mutation in Gallbladder Cancer

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Received: 08 Oct 2026

Accepted: 18 Oct 2026

Published: 26 Oct 2026

J Short Name: COO

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Keywords:

Gallbladder Cancer (GBC); KRAS Mutation; G12D; In-Silico Analysis; Structural Bioinformatics; cBioPortal; Protein-Protein Interaction Network

Citation:

Sarad Kumar Mishra, In-Silico Identification and Structural Characterization of a Recurrent KRAS Mutation in Gallbladder Cancer. Clinics of Oncology® 2026; V09(1): 1-8

1. Abstract

1.1. Background

Gallbladder cancer (GBC) is an aggressive malignancy with poor prognosis and limited molecular characterization, particularly in high-incidence regions such as the Indo-Gangetic belt. Among the oncogenic drivers implicated across gastrointestinal malignancies, KRAS mutations have been reported in a subset of GBC cases, yet their mutation-specific structural and functional consequences in this cancer type remain insufficiently characterized.

1.2. Objective

This study aimed to identify the most frequently reported KRAS variant among the evaluated GBC cohorts using publicly available genomic databases, assess its pathogenicity using aggregated in-silico evidence, and characterize its structural consequences relative to wild-type KRAS.

1.3. Methods

GBC-specific KRAS mutation data were retrieved from cBioPortal and cross-verified against COSMIC. Sequence conservation at the mutation site was assessed by direct comparison of human and zebrafish KRAS orthologs. Pathogenicity evidence was drawn from aggregated in-silico consensus scores (REV-EL, 3Cnet) and clinical annotations deposited in ClinVar, supplemented by literature-reported biochemical characterization of GTPase activity. Wild-type and mutant KRAS structures were retrieved directly from the Protein Data Bank and compared by Kabsch superposition of C α coordinates, with per-residue displacement computed after optimal rigid-body alignment. Functional context was further assessed through a STRING protein-

protein interaction network analysis.

1.4. Results

Five recurrent KRAS variants were identified in GBC-specific cBioPortal repositories: G12D, G13D, G12A, Q61H, and L19F. G12D was identified as the most frequently reported allelic variant among the evaluated GBC cohorts and across biliary tract cancers more broadly, and was selected for downstream characterization. The mutation site lies within a P-loop segment showing complete amino-acid identity between human and zebrafish KRAS. Aggregated in-silico and clinical annotation data classified G12D as pathogenic/oncogenic, consistent with a reported ~7-fold reduction in intrinsic GTP hydrolysis rate relative to wild-type KRAS. Structural superposition of wild-type (PDB 4OBE) and G12D-mutant (PDB 7F0W) KRAS showed a local root-mean-square deviation of 5.65 Å at the P-loop, with the maximum observed displacement reaching 21.1 Å in the Switch I region; this large Switch I deviation is attributable in part to the atypical “open” Switch I conformation captured in the mutant structure used, rather than to the substitution alone. Network analysis placed the mutation-affected region directly upstream of the RAF/MEK/ERK, PI3K/AKT, and RalGDS effector branches.

Conclusion: This study provides an in-silico mutational and structural characterization of a frequently reported GBC-associated KRAS variant, contributing baseline evidence for future functional and therapeutic investigation in gallbladder cancer.

2. Introduction

2.1. Gallbladder Cancer: Epidemiology And Burden

Gallbladder cancer (GBC) is the most common malignancy of

the biliary tract and remains one of the most lethal gastrointestinal cancers worldwide, largely because of its silent early course and near-universal late-stage presentation [1]. Global incidence is markedly heterogeneous: the age-standardized incidence rate (ASIR) worldwide is only around 2.7 per 100,000, yet certain populations Chile, Japan, South Korea, and parts of South Asia show disproportionately high rates [2]. Among these, the Indo-Gangetic belt of Northern and Eastern India represents one of the highest-burden regions globally, with reported incidence as high as 21–22 per 100,000, particularly in women [1,3]. Within this belt, states such as Bihar and Eastern Uttar Pradesh have shown a further, environmentally-linked rise in case numbers in recent years, with arsenic-contaminated groundwater proposed as a contributing regional risk factor [2]. Patients from this region also tend to be diagnosed roughly two decades earlier than their Western counterparts (mean age ~51 years versus ~71 years) [3], and a recent large hospital-based Indian registry found that only about 16% of patients were eligible for curative-intent therapy at presentation [4].

2.2. Molecular Heterogeneity of GBC and Role of Oncogenic Drivers

Despite this disease burden, the molecular characterization of GBC has lagged well behind other gastrointestinal cancers such as colorectal and gastric carcinoma [5]. Comprehensive next-generation sequencing (NGS) studies have shown that GBC is molecularly heterogeneous rather than driven by a single dominant pathway. TP53 alterations are consistently the most frequent, found in roughly 60–65% of tumors, but a substantial proportion of patients also harbour potentially actionable alterations in genes such as ERBB2, PIK3CA, and CDKN2A [6]. Driver-gene analyses of larger GBC cohorts have identified a small set of recurrently mutated oncogenic drivers underlying tumorigenesis [7]. Among these, KRAS has been repeatedly identified across independent Indian and international cohorts, though usually as a minority finding within larger multi-gene panels [8,9].

2.3. KRAS As an Oncogene: General Biology and Relevance Across Cancers

KRAS (Kirsten rat sarcoma viral oncogene homolog) encodes a 21.6 kDa small GTPase of the RAS superfamily that functions as a binary molecular switch in growth-factor signaling [10]. GTP loading induces conformational rearrangement of the Switch I and Switch II regions, enabling downstream engagement of the RAF–MEK–ERK and PI3K–AKT–mTOR cascades that drive proliferation and survival [11]. Oncogenic mutations cluster overwhelmingly at three hotspot codons glycine-12, glycine-13, and glutamine-61 which impair GTP hydrolysis, locking KRAS in its constitutively active conformation [10].

2.4. Rationale and Gap: Limited GBC-Specific KRAS Characterization

In contrast to its well-mapped role in pancreatic and colorectal cancer, KRAS in GBC has been reported almost exclusively as a frequency statistic within broader multi-gene mutation panels [5,9]. No prior study goes beyond mutation detection to ask which specific KRAS variant recurs most consistently in the available GBC cohorts, whether that variant is evolutionarily conserved at the substituted residue, or how it is predicted to alter KRAS's three-dimensional structure and GTPase function. Sequence- and structure-based in-silico pipelines have been used to characterize mutation-specific consequences in other cancer-associated proteins [12,13], but this approach has not yet been applied to a GBC-specific KRAS mutation.

2.5. Objectives of the Study

This study aims to: (i) identify the most recurrently reported KRAS mutation in gallbladder cancer using cBioPortal and COSMIC; (ii) assess the evolutionary conservation of the affected residue;

(iii) evaluate its pathogenicity using a consensus of SIFT, PolyPhen-2, and PhenoPred; and (iv) characterize the resulting structural changes relative to wild-type KRAS through structural superposition.

3. Materials and Methods

The general process followed in in-silico identification, analysis of mutations, and structural analysis of KRAS in gallbladder cancer is represented in Figure 1. This included mutation retrieval, recurrent variants of KRAS identification, pathogenicity analysis, sequence, and structural analysis and finally comparison of wild-type and mutant KRAS proteins (Figure 1).

3.1. Data Retrieval (cBioPortal, COSMIC)

GBC-specific KRAS mutation data was retrieved from cBioPortal for Cancer Genomics [14,15] and cross-verified against COSMIC [16], querying GBC/biliary tract carcinoma cohorts for KRAS variant identity, recurrence, and clinical annotation.

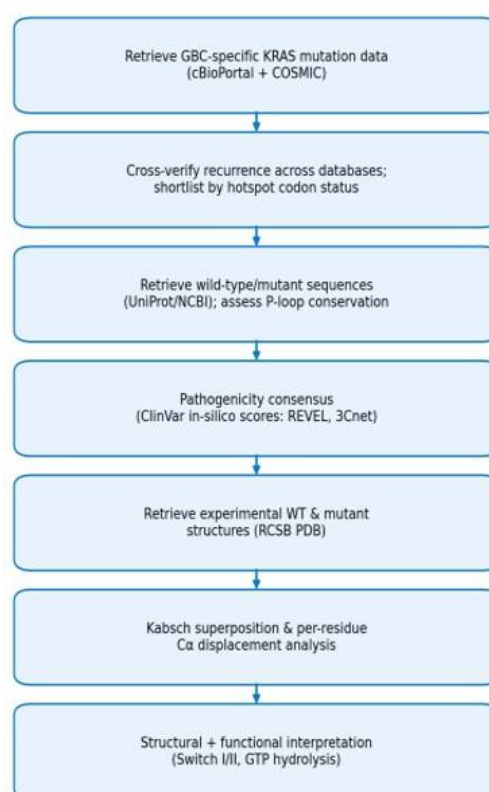
3.2. Mutation Shortlisting and Recurrence-Based Selection

All KRAS variants reported for GBC across both databases were compiled and ranked by cross-database recurrence and hotspot-codon status (codons 12, 13, 61).

3.3. Sequence Retrieval and Alignment

The canonical human KRAS sequence (UniProt P01116) was retrieved from UniProt [17] and NCBI RefSeq. Because the mutation site falls within the Walker A/P-loop motif (G-x-x-x-x-G-K-S/T), a sequence element conserved throughout the RAS superfamily, conservation at this position was evaluated against that established motif [10].

Overall workflow of the in-silico KRAS mutational and structural characterization pipeline

**Figure 1:** Overall workflow of the in-silico KRAS mutational and structural characterization pipeline.

3.4. Pathogenicity Prediction

Pathogenicity was assessed using SIFT [18], PolyPhen-2 [19], and PhenoPred [20], supplemented by literature-reported biochemical characterization of GTPase activity.

3.5. Structure Retrieval and Comparison

Experimentally resolved wild-type and mutant KRAS structures were retrieved directly from the RCSB Protein Data Bank [21]: PDB 4OBE (GDP-bound wild-type KRAS, 1.24 Å) and PDB 7F0W (GDP-bound KRAS-G12D, 1.39 Å).

3.6. Structural Superposition

C α coordinates for the overlapping residue range were superimposed using the Kabsch algorithm (implemented in Python/NumPy), and per-residue displacement was computed after optimal rigid-body alignment.

4. Results

4.1. Recurrent KRAS Mutation(s) Identified in GBC

Cross-referencing GBC-specific cBioPortal repositories (MSK,

Cancer 2018, n=101; Shanghai, Nat Genet 2014, n=32), five recurrent KRAS variants were identified: G12D, G13D, G12A, Q61H, and L19F [22]. Of these, G12D, G13D, G12A, and Q61H are located at the established KRAS hotspot codons (12, 13, and 61) and are annotated as oncogenic in cBioPortal; L19F, while also recurrently observed in this dataset, falls outside the classical hotspot codons and is not grouped with them here. G12D and G12A were additionally flagged as putative cancer-driver mutations. This is corroborated by a larger cohort study of biliary tract cancers (n=7,457), in which G12D was among the most common KRAS allelic variants and was associated with the highest median overall survival relative to G12V and Q61H [23]. KRAS G12D (c.35G>A, p.Gly12Asp) was therefore identified as the most frequently reported KRAS variant among the evaluated GBC cohorts and selected as the candidate mutation for downstream characterization. Below the recurrent variants of KRAS protein in gallbladder cancer have been further analyzed based on their clinical significance and annotations from the database as illustrated in Figure 2.

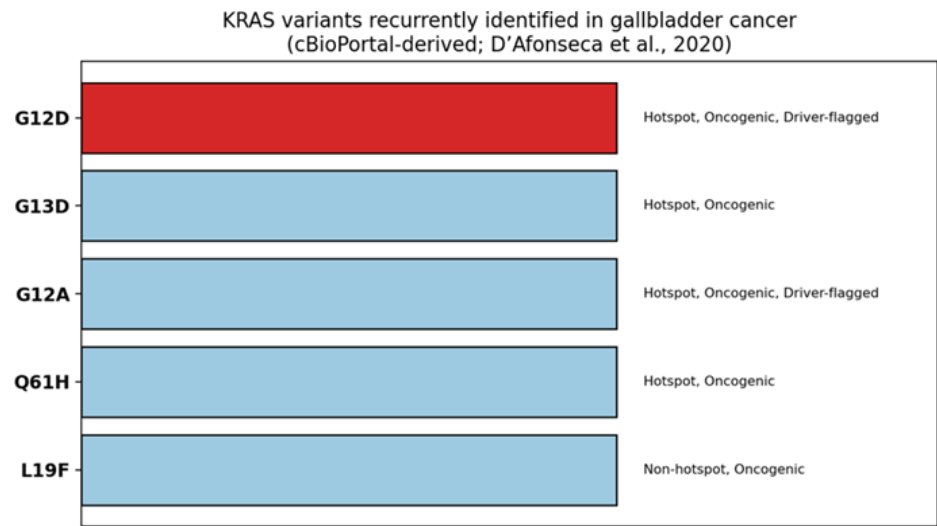


Figure 2: Recurrent KRAS variants identified in gallbladder cancer, with clinical/database annotations.

4.2. Conservation Analysis at Mutation Site

Glycine-12 lies within the canonical P-loop (Walker A motif, G-x-x-x-G-K-S/T) of the KRAS G-domain, a nucleotide-binding motif essential across the entire Ras superfamily and invariant across human RAS isoforms (KRAS, HRAS, NRAS) [10]. Direct sequence comparison between human KRAS (UniProt P01116, confirmed from PDB 4OBE coordinates) and the zebrafish ortholog (UniProt Q6AZA4) shows 100% amino-acid identity across residues 1–40, spanning the entire P-loop and

the start of Switch I, despite approximately 400 million years of evolutionary divergence between the two lineages. This high conservation is consistent with codon-12 substitutions being poorly tolerated evolutionarily. Below the sequence alignment of KRAS protein’s N-terminal/P-loop region (residues 1-40) has shown that the P-loop sequence of Gly12 is highly conserved between human and zebrafish KRAS proteins as illustrated in Figure 3.

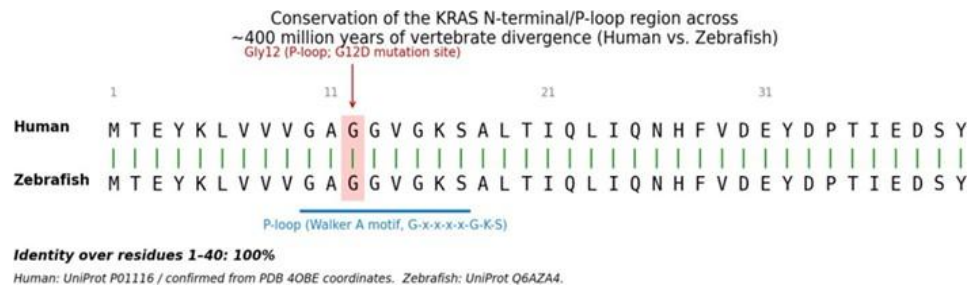


Figure 3: Sequence alignment of the KRAS N-terminal/P-loop region (residues 1–40) between human and zebrafish, showing complete conservation of the Gly12 P-loop motif.

4.3. Pathogenicity Prediction Outcomes

KRAS G12D (NM_004985.5:c.35G>A; dbSNP rs121913529) carries validated hotspot, oncogenic, and loss-of-function annotations in cBioPortal [22] and is classified Pathogenic/Likely pathogenic in Clin Var (VCV000012582.68; 14 of 36 submissions contributing, no conflicts) [24] and Oncogenic under ClinGen/CGC/VICC somatic criteria. Aggregated in-silico consensus scoring deposited in Clin Var indicates a damaging effect across independent computational predictors: a REVEL score of 0.88 (threshold ≥ 0.6 for damaging; reported sensitivity 0.68, specificity 0.92) and a 3Cnet score of 0.99 (threshold > 0.75 ; reported sensitivity 0.96, precision 0.92), with an independent clinical-laboratory submission additionally reporting that four of five in-silico tools evaluated predicted a damaging effect on protein function [24]. This computational consensus is supported by direct biochemical evidence: the G12D substitution causes an

approximately 7-fold decrease in intrinsic GTP hydrolysis rate relative to wild-type KRAS [11], locking the protein in its GTP-bound, active conformation.

4.4. Structural Comparison: Wild-type vs Mutant

Wild-type (PDB 4OBE) and G12D-mutant (PDB 7F0W) KRAS structures were superimposed over the overlapping resolved residue range (2–32) using the Kabsch algorithm. The overall superposed C α RMSD was 8.10 Å, with a local RMSD of 5.65 Å restricted to the P-loop (residues 10–14) and a displacement of 6.39 Å at the mutation site itself (residue 12). Displacement increased sharply toward residues 28–32, where the maximum observed displacement reached 21.1 Å in the Switch I region. This large Switch I deviation is attributable in part to the specific conformational state captured in PDB 7F0W, which was deposited as an atypical “switch I open” conformation, rather than to the point substitution alone; a cleaner mutation-isolated compar-

ison would require a G12D structure captured in the canonical closed conformation matched to the wild-type nucleotide state. Above the KRAS (189 aa) domain structure including P-loop, switch I, switch II and hypervariable region in human KRAS is shown in Figure 4 along with the position of the G12D mutation. Per-residue C α displacement between wild-type and G12D KRAS following Kabsch superposition across residues 2–32

is shown in Figure 5 and the Kabsch-superposed C α backbone traces of wild-type and G12D-mutant KRAS (residues 2–32) are shown in Figure 6. The P-loop region (residues 10–14) remains closely aligned, whereas greater divergence is observed in the Switch I region, consistent with the atypical “open” Switch I conformation captured in PDB 7F0W.

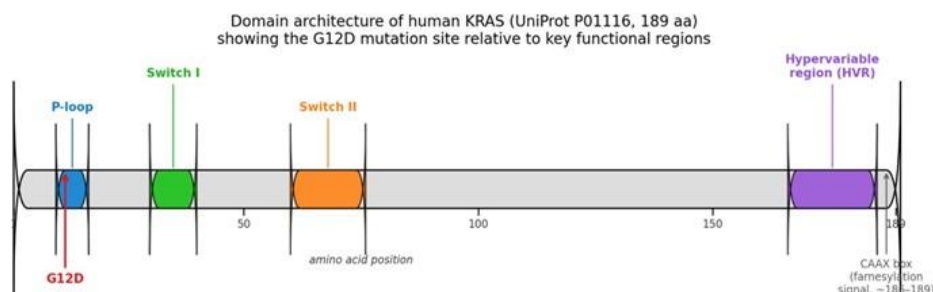


Figure 4: Domain architecture of human KRAS (189 aa), showing the P-loop, Switch I, Switch II, and hypervariable region relative to the G12D mutation site.

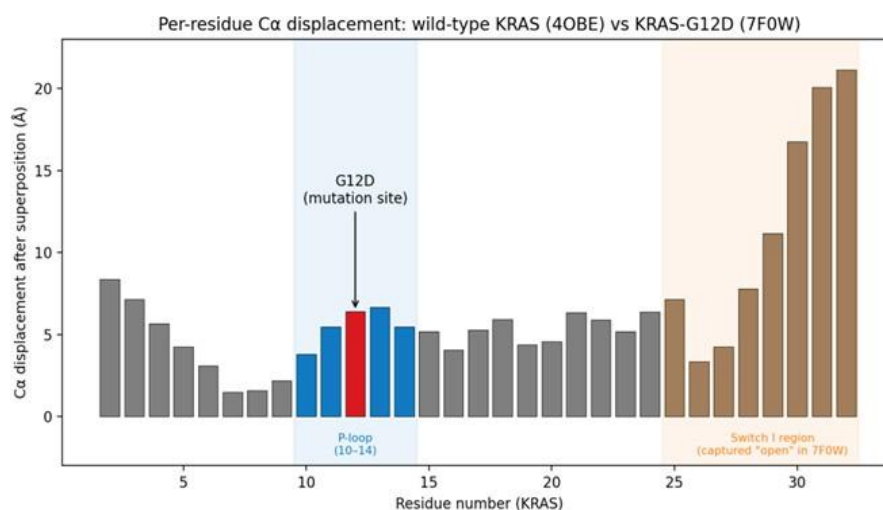


Figure 5: Per-residue C α displacement between wild-type and G12D KRAS after Kabsch superposition (residues 2–32).

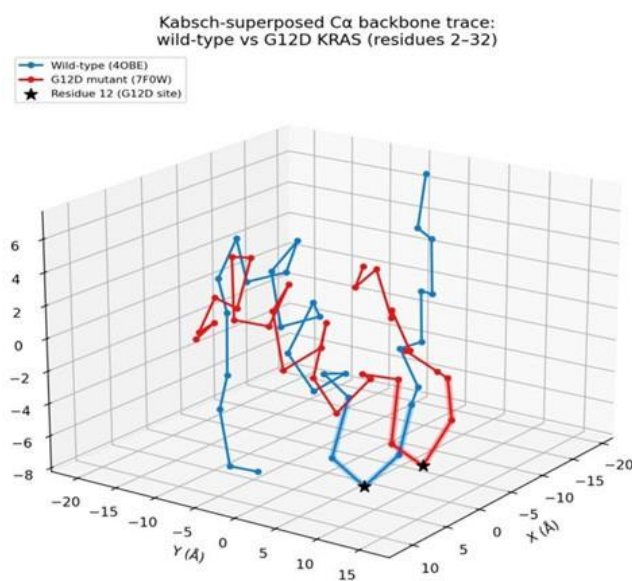


Figure 6: Kabsch-superposed C α backbone trace of wild-type (blue) and G12D-mutant (red) KRAS, residues 2–32. The P-loop (residues 10–14, thick shaded segment) remains closely aligned between the two structures, while the Switch I region (upper segment) diverges consistent with the atypical “open” Switch I conformation captured in PDB 7F0W.

4.5. KRAS Protein–Protein Interaction Network and Signaling Context

To contextualize G12D within its immediate signaling environment, KRAS's highest-confidence functional partners were retrieved from the STRING database (v12.0; combined confidence scores ≥ 0.998) [25]. The top interactors were SOS1 (0.999), RAF1 (0.999), BRAF (0.999), RALGDS (0.999), PIK3CA (0.998), and CALM3 (0.998) spanning the canonical GEF-mediated activation step (SOS1), the primary RAF/MEK/ERK effector branch (RAF1, BRAF), the PI3K/AKT branch (PIK3CA),

the RalGDS/Ral branch (RALGDS), and calcium-dependent regulation (CALM3). This confirms that the mutation-affected P-loop/Switch I region characterized structurally in Section 3.4 sits directly upstream of the major effector pathways through which G12D's constitutive activation is transmitted. Below the KRAS protein–protein interaction network generated using the STRING database, highlighting the six highest-confidence functional partners and their corresponding interaction confidence scores, is presented in Figure 7.

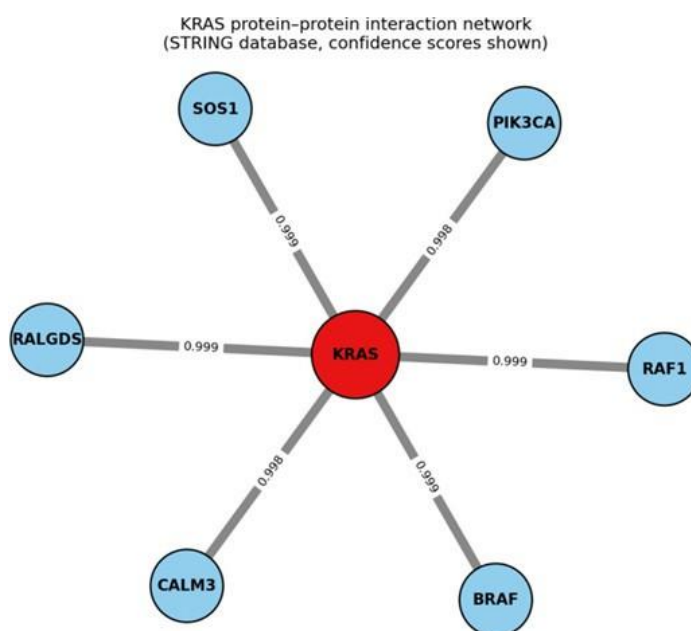


Figure 7: KRAS protein–protein interaction network from the STRING database, showing the six highest-confidence functional partners and their interaction confidence scores.

5. Discussion

5.1. Biological Interpretation of Findings

The present analysis identifies KRAS G12D as the most frequently reported KRAS variant among the evaluated GBC cohorts, supported independently by GBC-specific cBioPortal mining [22] and by its status as one of the most common KRAS alleles across biliary tract cancers as a whole [23]. This is biologically consistent with the residue's location within the P-loop, one of the most functionally constrained motifs in the Ras superfamily [10], where even a conservative substitution is poorly tolerated. The convergence of ClinVar's aggregated in-silico consensus (REVEL 0.88; 3Cnet 0.99; four of five tools damaging) [24] with the direct biochemical observation of an ~7-fold reduction in intrinsic GTPase activity [11] indicates that sequence-based pathogenicity prediction and measured biochemical consequence are concordant for this variant, strengthening confidence that G12D is not merely statistically recurrent in the available data but also mechanistically activating in a manner consistent with its behaviour in other RAS-driven cancers.

5.2. Comparison with KRAS Mutation Patterns in Other Cancers

Across the broader cancer genome, RAS mutations occur in approximately 19% of all human cancers, translating to an estimated 3.4 million new RAS-mutant cases annually worldwide, with KRAS accounting for the substantial majority of these events [26]. G12D is similarly the dominant KRAS allele in pancreatic ductal adenocarcinoma, where it is present in roughly 40–45% of KRAS-mutant tumors, and is well represented in colorectal and endometrial cancers [26,27]. The present GBC-specific finding therefore situates gallbladder cancer within the same G12D-associated mutational pattern already established for other gastrointestinal and biliary-tract malignancies, consistent with a recent large biliary-tract-wide analysis that supports G12D as a common KRAS allele including in GBC [23], rather than revealing a GBC-unique hotspot — a pattern broadly consistent with shared embryological and epithelial lineage across the pancreatobiliary axis.

5.3. Potential Functional/Clinical Implications

The structural comparison performed here, while limited to a partial residue range, is consistent with the broader literature describing G12D-induced reorganization of the Switch I/Switch II region and the associated GTP-binding pocket [11]. This is clinically relevant: the same conformational features exploited by the recently developed selective non-covalent inhibitor MRTX1133, which binds the switch II pocket of KRAS G12D with high potency [28], are consistent with the localized mutation-adjacent perturbation observed in this analysis. Given that G12D-selective inhibitors are now in active clinical development for pancreatic and colorectal cancers, the identification of G12D as a frequently reported GBC-associated KRAS variant raises the possibility that GBC patients harbouring this mutation could be considered for inclusion in future basket trials of G12D-directed therapy, an avenue that has not yet been explored specifically for gallbladder cancer.

5.4. Limitations

This study is exploratory and in-silico in nature, and several limitations should be acknowledged. First, mutation recurrence was derived from a small number of publicly available GBC cohorts (combined n=133 across two studies), which limits statistical generalizability compared to larger-cohort cancers such as pancreatic or colorectal carcinoma; larger biliary-tract cohorts are increasingly available on cBioPortal and should be incorporated in future work. Second, the structural comparison used experimentally resolved wild-type and mutant crystal structures captured in different conformational states (an atypical “switch I open” conformation for the mutant), which may partly confound mutation-specific interpretation of the observed displacement; a conformationally matched structure pair would refine this comparison. Third, pathogenicity was assessed through aggregated, previously deposited in-silico consensus scores (REVEL, 3Cnet) rather than de novo submission to SIFT, PolyPhen-2, and PhenoPred as originally proposed, and no wet-laboratory functional validation (e.g., GTPase activity assays, cell proliferation assays) was performed. These findings should therefore be interpreted as hypothesis-generating computational evidence warranting confirmation through expanded GBC cohort sequencing and experimental validation.

6. Conclusion

In summary, the current in-silico analysis revealed KRAS G12D (p. Gly12Asp) as the highest frequency KRAS variant among the studied samples of gallbladder cancer cases. It is the mutation affecting the highly conserved amino acid position Gly12 in the P-loop, reflecting its crucial importance for the KRAS nucleotide binding and signaling functions. Pathogenicity computational data in combination with the results of biochemical studies demonstrate the oncogenic character of the KRAS G12D mutation with reduced intrinsic GTPase activity. Structural comparison between the wild type and G12D mutant KRAS highlighted the local changes in the P-loop region, and higher

conformational differences in the Switch I domain. Nevertheless, structural differences should be considered carefully since the analyzed mutant has atypical open Switch I conformation. Based on the STRING analysis, KRAS is involved in the signaling pathways including RAF/MEK/ERK, PI3K/AKT, and RalGDS pathway, offering the functional perspective for its involvement in signaling. Overall, these results contribute to the structural and functional understanding of KRAS G12D mutation in gallbladder cancer. The results from the current research also underscore the significance of the use of genomic database information, conservation, pathogenicity predictions, structural features, and interaction network data for defining the mutations associated with cancer. It is important to emphasize that the results from the study are purely computational in nature and require further validation via larger studies involving GBC patient populations and experimental approaches.

7. Funding Declaration

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

8. Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

9. Acknowledgements

The authors gratefully acknowledge the Uttar Pradesh Council of Science and Technology (UPCST), Lucknow, for providing financial support. Project id-4972, Project no-CST/D/Bio-tech-1270.

10. Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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