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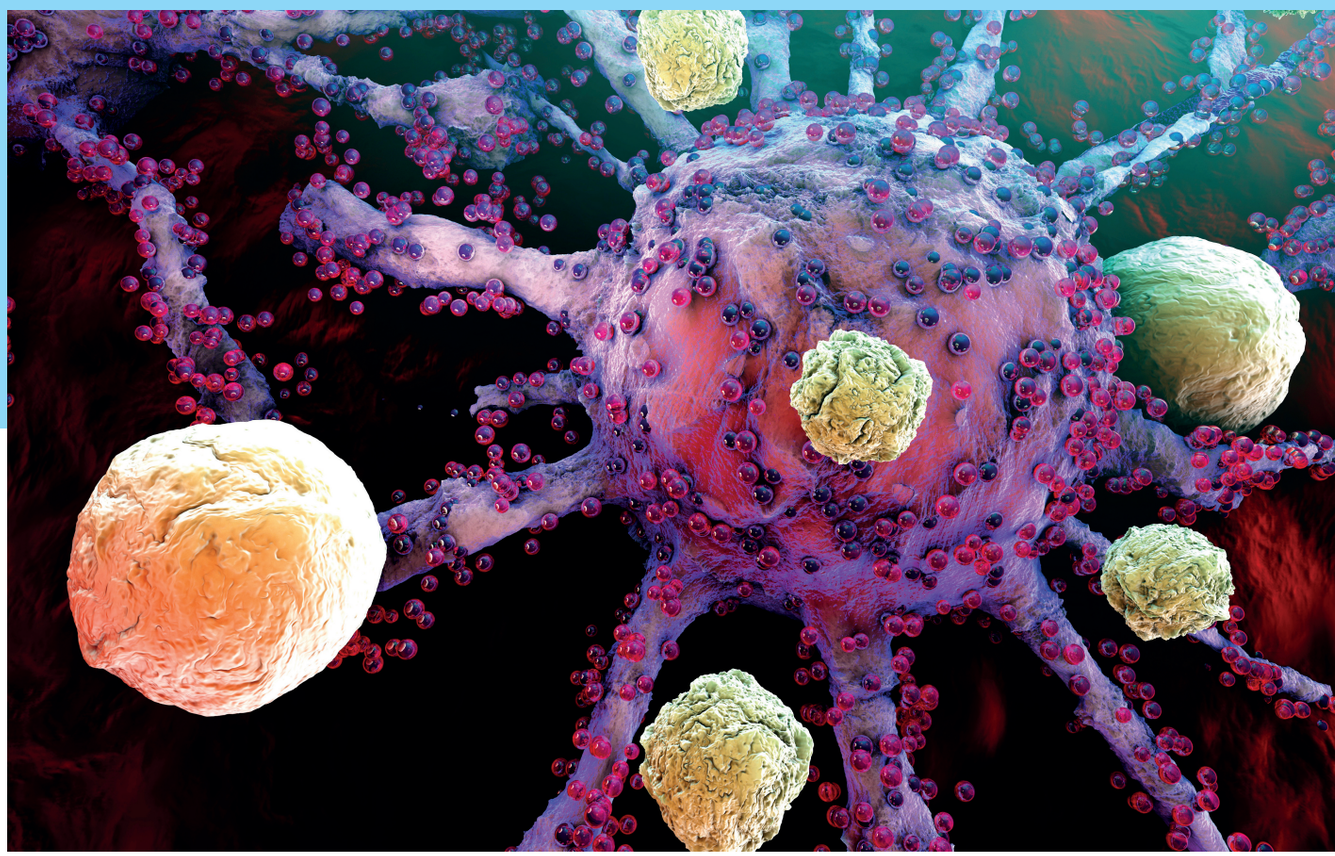

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Expert Consensus on Diagnosis and Molecular Testing Strategies for Non-Small-Cell Lung Cancer in India

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Abstract

This review aims to establish expert consensus on biomarker testing for non-small-cell lung cancer (NSCLC) in India, evaluating diagnostic practices, adherence to international guidelines, and test utility and comparing clinically validated assays with laboratory-developed tests. In round 1, experts voted on 41 statements covering various aspects of NSCLC diagnostics. Responses were graded using a 5-point Likert scale, categorizing agreement levels as high, moderate, or low based on expert consensus percentages. After thorough deliberations during round 2, consensus was reached on 32 statements underscoring the necessity for early diagnosis of NSCLC. Key issues include misdiagnosis with tuberculosis and low or delayed specialist referral rates. Although formal programs are limited by awareness, resources, and data gaps; low-dose computed tomography (LDCT) screening in community settings is advocated. Consensus was reached among experts that most lung cancers are diagnosed at advanced stages in India. The delay in diagnosis was mainly due to misdiagnosis with tuberculosis and delayed referrals to specialists for evaluation. The consensus acknowledged the need to enhance lung cancer awareness and utilization of LDCT in high-risk individuals as a screening methodology in the community.

Biomarker testing for both early-stage and advanced-stage NSCLC is recommended, with reflex testing at diagnosis, longitudinal testing at disease progression, and liquid biopsies when tissue is unavailable/inadequate. Biomarker testing for common driver mutations associated with available targeted therapies can be performed in resource-

Keywords
- non-small-cell lung cancer
- next-generation sequencing
- biomarkers
- companion diagnostic
- reflex testing
- targeted therapies
- low-dose computed tomography

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limited settings using sequential testing or hotspot panels. PD-L1 testing for all advanced-stage cases is recommended, especially when molecular driver mutations/fusions are negative. Despite longer turnaround times, next-generation sequencing (NGS) would be preferred for its comprehensive gene assessment. Multigene assays are recommended for advanced stages, and upfront broad-panel tests are ideal. Testing for *EGFR*, *ALK*, proto-oncogene ROS1, and PD-L1 is essential for NSCLC. We urge standardized histopathological and molecular practices and acknowledge challenges in NGS availability and the complexities of interpreting results. This consensus underscores the importance of streamlined approaches to enhance NSCLC diagnostics in resource-constrained settings in India.

Introduction

Lung cancer ranks as the most commonly diagnosed cancer worldwide, accounting for 2.48 million new cases in 2022, with an associated mortality of 1.8 million deaths annually.[1,2] In India, the crude incidence rate of lung cancer is 7.1 cases per 100,000 people, with projections indicating a likely increase in the coming years.[3] Non-small-cell lung cancer (NSCLC) constitutes approximately 85% of all lung cancer cases globally.[4] Among the subtypes of NSCLC, adenocarcinoma is the most prevalent, representing approximately 40% of all lung cancer cases.[5] Adenocarcinomas account for approximately 34.3% of lung cancers in males and 52.7% in females, while squamous cell carcinomas constitute 11.5% of all lung cancer cases in India.[6] Most NSCLC cases (>90%) are detected late, specifically in stage III or IV, limiting curative options. Only 31.7% of patients with stage I–IIIb NSCLC receive surgery or radical radiotherapy.[7–9] While stage IV is often managed with palliative care, stage III can be treated with curative intent. The 5-year survival rate of lung cancer stands at 63.7%. Most cases are diagnosed after metastasis, highlighting the need for early detection and treatment.[10]

The selection of treatment is influenced by the disease stage, the patient's overall health, and the presence of specific biomarkers.[4] To ensure the effectiveness of targeted therapies in patients with advanced-stage NSCLC, molecular analyses are standard for identifying genetic mutations or rearrangements, including *EGFR*, *ALK*, proto-oncogene ROS1, and others presented in ►**Supplementary Table S1** (available in the online version only).[11–13] Biomarker identification enables targeted therapies and immunotherapy to be directed to the most suitable patients, improving treatment outcomes.[14]

International guidelines from the National Comprehensive Cancer Network (NCCN), European Society for Medical Oncology (ESMO), and International Association for the Study of Lung Cancer (IASLC) emphasize the importance of comprehensive molecular testing for NSCLC. Although the guidelines highlight the importance of conventional methods for single-gene testing, such as polymerase chain reaction (PCR), immunohistochemistry (IHC), and fluorescence in situ hybridization (FISH), to assess specific biomarkers, next-generation sequencing (NGS) and comprehensive genomic profiling are advocated for providing a broader analysis.[15–18]

The evolving NSCLC diagnostic landscape requires continuous updates to integrate new biomarkers but is hindered by operational and logistical challenges.[19] The 2019 Indian consensus guidelines primarily emphasize early screening and detection of NSCLC and biomarker testing for advanced cases.[19] Since then, early- and advanced-stage NSCLC management advancements have necessitated revisions. While these guidelines are based on scientific evidence, practical factors—such as affordability and availability of tests and drugs, out-of-pocket costs, and insurance coverage—must also be considered.[11] Additionally, testing for emerging resistant mutations is essential for patients resistant to *EGFR* tyrosine kinase inhibitors (TKIs).

This modified Delphi consensus aimed to achieve the following objectives:

- To investigate the role of biomarkers in diagnosing early- and advanced-stage NCSLC.
- To assess the applicability of international guidelines (such as those from the NCCN, ESMO, and IASLC) for NSCLC diagnostics in the Indian context.
- To evaluate the utility and feasibility of various diagnostic tests, such as NGS, in managing NSCLC in resource-constrained settings in India.
- To determine the accuracy and reliability of various clinically validated assays compared with laboratory-developed tests in the Indian context.

Materials and Methods

Study Design

This study used the modified Delphi method to solicit input from a select panel of experts specializing in NSCLC diagnostics. This iterative and structured approach aims to synthesize the collective expertise of a diverse panel of experts to achieve consensus on complex or uncertain topics.[20]

Panel of Experts

The expert panel comprised four oncologists and four pathologists from various institutions, each recognized as a leader in medical oncology, precision oncology, oncopathology, histology, and cytology. The panel members brought experience from public and private health care sectors across different regions of India, ensuring a well-rounded perspective that

accounted for variations in clinical practices and health care infrastructure.

Delphi Questionnaire Development

The modified Delphi process entailed an initial round of expert voting on the 41 statements. Statements were graded based on available evidence from the literature,[21] with responses recorded on a 5-point Likert scale (strongly agree, agree, neither agree nor disagree, disagree, and strongly disagree). Consensus levels were categorized as "high" ($\geq$75% agreement), "moderate" (55–74% agreement), or "low" ($<$55% agreement). Open-ended responses were collated and summarized.

A total of 41 statements spanning the following thematic domains were voted upon and deliberated by the experts:

- Challenges in early diagnosis of NSCLC.
- Diagnosis and biomarker testing in NSCLC.
- NGS testing in NSCLC.
- Practical considerations in NSCLC management.

The experts were provided access to the consensus statements and questionnaire on a proprietary platform via an electronic link. They comprehensively examined the challenges, considerations, and recommendations pertinent to NSCLC management pathways in the Indian context.

Two-Round Modified Delphi Process

Subsequently, statements without consensus in round 1 were discussed in a face-to-face meeting during round 2. Here, statements were refined, added, or removed based on expert discussion. A final consensus was reached on statements with high agreement levels.

Results

In the first round of voting, 41 statements were evaluated: 24 statements achieved a high level of agreement, 15 showed a moderate level of agreement, and two statements presented as open-ended questions did not fall into either category. During the discussion, statements that required additional clarification were determined. These statements were revised and reworded in the second round. These modifications resulted in a more substantial consensus among the experts. By the conclusion of round two, 32 statements were finalized, each attaining a high level of consensus. Several statements were further reframed for clarity.

Discussion

The discussion synthesizes expert insights on critical themes in NSCLC care, including early diagnosis, biomarker and NGS testing, and practical management considerations, with a focus on real-world challenges and practices in India.

Challenges in the Early Diagnosis of NSCLC

Early diagnosis of NSCLC is critical for enabling prompt treatment, which can lead to improved outcomes, higher cure rates, and enhanced survival rates.[22] Five statements on the early diagnosis of NSCLC attained high consensus (►**Table 1**).[22–24]

A retrospective analysis (2008–2016) of 1,370 patients with NSCLC in India revealed that 95% were diagnosed at advanced stages, with considerable delays in obtaining a definitive diagnosis. Misdiagnosis is a major contributing factor to delayed NSCLC diagnoses due to its overlapping symptoms with endemic tuberculosis.[22] Additionally, insufficient awareness about the early signs and risk factors of lung cancer contributes to delayed detection.[25]

Low-dose computed tomography (LDCT) screening has proven reliable in the early identification of NSCLC in high-risk individuals. As per the NCCN guidelines, annual LDCT screening is advised for individuals at high risk for lung cancer.[26] The National Lung Screening Trial demonstrated a reduction in mortality among high-risk individuals who underwent LDCT compared with those who underwent standard chest X-ray screenings.[27,28] Barriers to adopting LDCT include a limited understanding of its effectiveness and advantages and inadequate infrastructure.[29] Despite the high

Table 1 Statements on screening for NSCLC

Statemen t No.	Statement	Level of evidence	Consensus achieved
1.	In India, most patients with lung cancer are diagnosed at an advanced stage, which emphasizes the need for early diagnosis.[22]	3b	75%
2.	Misdiagnosis plays a major role in the late diagnosis of NSCLC due to overlapping symptoms with endemic tuberculosis.[22]	3b	87%
3.	Low referral of patients to specialist centers could contribute to a delayed diagnosis of NSCLC.[22]	3b	87%
4.	LDCT screening for high-risk individuals, especially in resource-limited nations, may substantially help diagnose NSCLC early.[23]	2b	75%
5.	There is no formal screening program that includes LDCT for patients with lung cancer due to the unavailability of clinical data in the Indian population.[24]	5	100%

Abbreviations: LDCT, low-dose computed tomography; NSCLC, non-small-cell lung cancer.

incidence of lung cancer in India, there are no structured national screening programs in place.[30] Resource limitations, low awareness among health care providers, and inadequate infrastructure pose substantial challenges, hindering early lung cancer diagnosis. Key obstacles also include high false-positive rates (mainly due to the high prevalence of tuberculosis) and reluctance among high-risk individuals to undergo screening.[24,31]

A pilot retrospective study in India involving 350 smokers who underwent LDCT screening found lung nodules in 93% of individuals, with category two nodules being the most common (41%) and category four nodules found in 14%. Seven patients with category 4 nodules were diagnosed with lung cancer (six adenocarcinomas and one small cell lung cancer), resulting in an overall cancer incidence of 2%. This demonstrates that LDCT can effectively detect malignant lung nodules even in a tuberculosis-endemic country.[32] Experts recommend that LDCT should ideally be integrated into routine screening in India. Given existing limitations, its implementation should be phased, beginning with high-risk individuals.

Diagnosis and Biomarker Testing in NSCLC

All statements on the diagnosis and biomarker testing for NSCLC achieved high consensus (►**Table 2**).[11,19,33–47]

Histological assessment guides initial diagnosis and obtaining adequate biopsy samples is pertinent. Tissue

Table 2 Statements on diagnosis and biomarker testing in NSCLC

Statement No.	Statement	Level of evidence	Consensus achieved
6.	Reflex biomarker testing, where the pathologist initiates biomarker testing at the time of initial NSCLC diagnosis, should be strongly considered over testing after consultation with an oncologist or a multidisciplinary team.[33,34]	3b	87%
7.	Diagnostic utility and clinical validity play a major role in IHC testing.[35]	3b	100%
8.	Single biomarker testing is commonly adopted in resource-limited settings.[36]	5	100%
9.	Testing for *EGFR*, *ALK*, and PD-L1 is suggested for patients with early-stage NSCLC.[19]	5	100%
10.	Testing for *EGFR*, *ALK*, and *ROS1* is strongly advisable for all patients with advanced-stage NSCLC, irrespective of histologic subtype, for treatment decision-making.[19]	5	100%
11.	In a relatively asymptomatic patient, it may be advisable to wait for molecular testing results before choosing the optimal first-line treatment.[37]	5	100%
12.	EGFR testing is recommended for all stages of NSCLC, as it is the most common driver mutation in patients with lung cancer in India.[38]	2b	100%
13.	T790M testing should be performed after progression on first-line first- and second-generation TKIs.[11]	1a	100%
14.	For T790M testing, a liquid biopsy is preferable; however, if the results are negative, a tissue biopsy should be performed.[11,39]	3b	100%
15.	Liquid biopsy can be used as a complementary test to tissue biopsy.[40]	2b	100%
16.	In diagnosed cases of NSCLC, liquid biopsies that assess mutations in circulating DNA offer less-invasive alternative(s) for biomarker testing.[41]	3b	100%
17.	ALK IHC is a US FDA-approved companion diagnostic for various ALK inhibitors with high concordance with FISH and NGS.[42,43]	3b	100%
18.	ROS1 IHC is a valuable screening test, but positive cases need to be confirmed with FISH/NGS.[44]	2a	100%
19.	Longitudinal testing (re-biopsy at progression) is key to guiding treatment decisions by identifying tumor evolution, resistance pathways, and new molecular alterations.[45]	3b	100%
20.	PD-L1 testing is recommended for all NSCLC cases, irrespective of the stage.[11]	1a	100%
21.	For PD-L1 testing in NSCLC, the SP263 assay can be used as an alternative to the DAKO platform, given the strong concordance between the SP263 and 22C3 clones.[46]	1a	87%
22.	22C3 testing is preferable for treatment with pembrolizumab.[47]	2b	100%
23.	There is a need for homogenization of histopathological and molecular practices among pathologists regarding appropriate tissue triaging and reporting of various indicators (tissue adequacy, tumor microenvironment, necrosis, background cellular component, etc.) that influence downstream molecular testing and its interpretation.[19]	5	100%

Abbreviations: ALK, anaplastic lymphoma kinase; DNA, deoxyribonucleic acid; *EGFR*, epidermal growth factor receptor; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; NGS, next-generation sequencing; NSCLC, non-small-cell lung cancer; PD-L1, programmed death-ligand 1; *ROS1*, proto-oncogene tyrosine-protein kinase; T790M, threonine-to-methionine substitution at amino acid position 790; TKI, tyrosine kinase inhibitor; US FDA, United States Food and Drug Administration.

biopsy remains the gold standard for NSCLC diagnosis, in classifying tumors and identifying actionable mutations. Optimal tissue samples should be obtained to ensure accurate histological diagnosis and molecular testing, enabling personalized treatment decisions.[18] Tissue samples should adequately represent the tumor's cellular and microenvironmental characteristics. Since tissue requirements vary by assay, prioritization is necessary to optimize its use.[48]

Molecular testing for "druggable" mutations is essential in enhancing diagnostic precision, guiding targeted therapies, and improving treatment outcomes.[19] The various diagnostic methods employed for single-gene testing include PCR, IHC, FISH, and NGS.[35] In resource-constrained health care systems, such as India, the reliance on sequential single-gene testing persists due to cost and accessibility limitations.[49] Reflex biomarker testing should be considered at diagnosis.[33,34]

While PCR is a widely used method for detecting *EGFR* mutations,[17] IHC is recommended for *ALK*, *ROS1*, and PD-L1 testing. IHC is a reliable tool for detecting *ALK* rearrangements and *ROS1* fusions in NSCLC.[43] Literature suggests that IHC is a robust, cheap, and widely available method and an essential diagnostic test for *ALK* rearrangements in NSCLC. Alternatively, FISH can be used for *ALK* rearrangement detection. Cases with equivocal staining can be further confirmed with FISH.[43] However, FISH requires greater technical expertise and is expensive.[43] The *ALK* (D5F3) IHC is a U.S. Food and Drug Administration-approved companion diagnostic assay and does not need further confirmation by FISH. Results from a concordance study comparing various IHC antibodies with FISH as the gold standard showed that IHC using D5F3 and 5A4 clones is rapid and cost-effective for detecting various *ALK* gene rearrangements.[50] IHC is an important screening method for *ROS1* fusions. However, positive cases must be confirmed with FISH or NGS.[17]

While tissue-based biopsy remains essential for diagnosis, it is expensive. Other disadvantages of tissue-based biopsies include the need for an invasive procedure, challenges with certain tumor locations, limited tissue availability, and difficulty in capturing tumor heterogeneity.[40] Additionally, single-gene testing may require sequential tests, which can be compromised by limited tissue availability. Multiple tests may also result in treatment delays.[51,52]

Liquid biopsy can be recommended where tissue is inadequate for molecular testing or re-biopsy is not feasible.[53] They can also be used in cases of progression after TKI treatment (*EGFR*, *ALK*) or when faster results are needed. Liquid and tissue biopsies complement each other by identifying more actionable genomic alterations when used together than individually.[40] Liquid biopsy can overcome intratumor or inter-tumoral heterogeneity for the detection of molecular alterations.[54] Liquid biopsies enable detection by analyzing circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs).[53] The analysis of CTCs in monitoring treatment response and prognosis is currently restricted to clinical trials.[55] Additionally, liquid biopsy may be utilized to monitor residual disease after surgery and assess early molecular response to targeted therapy through ctDNA clearance, although it remains experimental.[53,56,57] The ESMO guidelines recommend liquid biopsy in patients ineligible/unwilling for repeat biopsy or when tissue samples are insufficient.

Similarly, the NCCN guidelines advise performing a liquid biopsy to detect the threonine-to-methionine substitution at amino acid position 790 (T790M) mutation in cases with cancer progression.[54] Along with advantages such as minimal invasiveness, patient acceptability, high repeatability, and reduced costs, the sensitivity of liquid biopsies in detecting the T790M mutation is comparatively higher than that of tissue biopsies.[54] Moreover, liquid biopsies or re-biopsies play a valuable role in detecting new gene alterations during NSCLC progression and in detecting histological changes.[55,58]

In early-stage NSCLC, testing for *EGFR* mutations, *ALK* fusion, and PD-L1 expression is recommended, as these are common driver mutations and have targeted therapies and immunotherapies available.[38,59–61]

PD-L1 testing can be considered in all cases of NSCLC, regardless of stage. However, experts opined that in early-stage, resectable NSCLC, the role of PD-L1 testing is limited when using perioperative chemo-immunotherapy approaches.[62] Similarly, in stage III NSCLC, durvalumab is approved as maintenance therapy post-chemoradiotherapy (CTRT) in India, irrespective of PD-L1 status.[12] Higher PD-L1 expression in immune cells is associated with improved overall survival in patients.[63,64] PD-L1 testing is primarily conducted using IHC. The tumor cell expression score is highest when using the 22C3 antibodies compared with assays such as SP142. This makes 22C3 testing preferable as it reliably identifies patients eligible for pembrolizumab therapy.[47]

Experts reiterate that early diagnosis, followed by timely biomarker testing, can notably improve survival outcomes by enabling the selection of appropriate targeted therapies and immunotherapies.[38,59–61]

In India, approved treatments include erlotinib, dacomitinib, afatinib, gefitinib, and osimertinib for *EGFR* mutations; crizotinib, alectinib, ceritinib, and lorlatinib for *ALK*; and entrectinib for *ROS1*. ►**Supplementary Table S1** (available in the online version only) lists the different testing methods categorized as mandatory, preferable, and optional biomarkers with the approved drugs.

NGS Testing in NSCLC

NGS-based assays, which analyze multiple biomarkers simultaneously, have demonstrated good clinical utility in NSCLC diagnostics.[65] Evidence suggests that RNA-based NGS assays effectively detect gene rearrangements and fusions, and combining them with DNA-NGS enhances the identification of clinically relevant mutations.[66,67]

Broad-panel NGS is preferred over sequential single-gene testing to guide treatment decisions when feasible and affordable.[65,68]

Despite its longer turnaround time, NGS offers a faster, more comprehensive approach.[51,52] Although NGS has a higher initial cost, it can be more cost-effective over time by focusing on clinically relevant biomarkers[65] and minimizing the need for multiple tests.

In India, NGS remains underutilized due to cost constraints and limited availability.[69] However, experts highlighted that cost-effectiveness could be improved through strategic panel selection. Challenges persist, including a lack of clinical validation and standardization of NGS panels. Limited laboratory infrastructure and a shortage of trained personnel are other barriers. Generating clear NGS reports and adhering to best practice guidelines are crucial for ensuring accurate interpretation and high-quality outcomes.[65,70]

Experts noted variations in pathologists' practices due to the absence of guidelines in India, emphasizing the need for standardization. Reporting of indicators such as the quality and adequacy of tissue for testing, and its cellular components were of utmost importance in optimizing downstream molecular testing and its interpretation.

Consensus statements regarding NGS testing are listed in ►**Table 3**.[11,71–75]

Practical Considerations in NSCLC Management

Reflex testing initiated by pathologists can expedite biomarker testing, ensuring broader coverage.[35] Our expert consensus underscores the importance of tissue adequacy for reflex testing and prioritizing molecular diagnostic assays in managing NSCLC. We advocate for the wider adoption of reflex testing practices, particularly in India, to enhance diagnostic efficiency.

Furthermore, considering the affordability and accessibility of tests and drugs, we recommend incorporating a broad-panel NGS testing along with PD-L1 IHC, where feasible.

It is important to ensure an adequate tissue sample for testing and consider repeating biopsies as necessary. When tissue samples are insufficient for comprehensive molecular testing, alternative methods such as IHC and FISH can be employed to assess *ALK* and *ROS1*. These techniques require smaller tissue samples and can provide information on genetic alterations. Timely assessments by pathologists and accurate labeling for molecular testing are essential to maintain diagnostic precision. Traditionally, biomarker tests for lung cancer are ordered by the treating oncologist only after a confirmed pathological diagnosis, which can lead to delays in treatment. Finally, comprehensive technician training and the establishment of in-house genomic protocols to enhance the efficiency of molecular testing for NSCLC diagnosis are recommended. Standardizing practices among pathologists for tissue triaging and identifying tissue adequacy that impacts molecular testing is essential. Improved communication and multidisciplinary collaboration among radiologists, pulmonologists, pathologists, and oncologists to advance patient care in NSCLC management are urgently needed.

Table 3 Statements on NGS testing in NSCLC

Statement No.	Statement	Level of evidence	Consensus achieved
24.	In India, limited in-house availability and affordability limit the use of NGS technology, and there is a need for its clinical validation.[71]	3b	100%
25.	NGS testing is associated with the longest average turnaround time as compared with other methods, such as PCR, qPCR, IHC, and FISH.[36]	5	72%
26.	There is a high concordance between clinically validated multigene NGS panels and single-gene assay for detecting *EGFR* mutations and *ALK* fusions.[72]	3b	87%
27.	Compared with serial single-gene testing, NGS testing at CAP/CLIA/NABL-accredited laboratories to investigate multiple genes in a single run is preferable, timesaving, and cost- effective.[73]	3b	100%
28.	NGS assays enable the assessment of multiple genes simultaneously with high sensitivity while saving time and tissue compared with sequential single-gene testing. However, these assays are complex in design, performance, and interpretation.[74]	3b	87%
29.	Targeted hotspot panel testing is preferable over CGP in resource-constrained settings due to affordability and availability.[11]	1a	100%
30.	Multigene assays can be recommended upfront for advanced-stage NSCLC (stages IIIb–IV).[11]	5	100%
31.	Broad-panel tests should ideally be performed upfront, but if missed, they should be performed later.[75]	3b	100%
32.	RNA-based NGS panels are recommended for detecting fusions involving *ALK*, *ROS*, *RET*, *NTRK*, and *NRG1* genes, preferably from FFPE samples.[74]	3b	87%

Abbreviations: ALK, anaplastic lymphoma kinase; CAP, College of American Pathologists; CGP, comprehensive genomic profiling; CLIA, clinical laboratory improvement amendments; *EGFR*, epidermal growth receptor factor; FFPE, formalin-fixed paraffin-embedded; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; NABL, National Accreditation Board for Testing and Calibration Laboratories; NGS, next-generation sequencing; *NRG1*, neuregulin-1; NSCLC, non-small-cell lung cancer; *NTRK*, neurotrophic receptor tyrosine kinase; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; RET, rearranged during transfection; RNA, ribonucleic acid; *ROS1*, proto-oncogene tyrosine-protein kinase.

Table 4 Key recommendations on diagnosis of NSCLC in India

Early diagnosis is vital for patients with lung cancer in India, where most cases are advanced. LDCT screening in communities can aid early NSCLC detection while improving referrals to specialist centers, which is key to avoiding diagnosis delays.
For molecular testing, *EGFR*, *ALK*, and PD-L1 testing should be prioritized in both early-stage and advanced-stage NSCLC cases to guide treatment decisions. In addition, *ROS1* testing needs to be prioritized in advanced-stage NSCLC.
T790M testing is recommended after progression on first-line first- or second-generation *EGFR*-TKIs, starting with liquid biopsy, followed by tissue biopsy if liquid biopsy results are negative.
PD-L1 testing is essential for treatment decision-making in advanced-stage NSCLC and should be considered for early-stage cases as well. The PD-L1 SP263 assay can be used if the DAKO platform is unavailable for PD-L1 testing.
Reflex biomarker testing at initial diagnosis is recommended to expedite treatment initiation.
Multigene assays can be recommended upfront for advanced-stage NSCLC, as they offer greater efficiency and cost-effectiveness compared with serial single-gene testing.
Efforts should focus on improving the availability and affordability of NGS technology in India, as it is preferable for comprehensive gene analysis in advanced-stage NSCLC.

Abbreviations: ALK, anaplastic lymphoma kinase; EGFR, epidermal growth factor receptor; LDCT, low-dose computed tomography; NGS, next-generation sequencing; NSCLC, non-small-cell lung cancer; PD-L1, programmed death-ligand 1; ROS1, proto-oncogene tyrosine-protein kinase; TKI, tyrosine kinase inhibitor; T790M, threonine-to-methionine substitution at amino acid position 790.

The strengths of this review include the use of a structured modified Delphi method, involvement of a multidisciplinary expert panel, and their representation from diverse health care settings across India. However, as with any consensus-based review, the findings may not fully reflect evolving clinical practices or future developments. The recommendations are particularly applicable to health care settings with limited resources. Persistent gaps in evidence, particularly related to early diagnosis, biomarker testing workflows, and NGS implementation, warrant further research and validation in real-world settings.

The key recommendations from this consensus are provided in ►**Table 4**.

Conclusion

This review highlights the need for enhancing lung cancer awareness and introducing formal screening programs, specifically using LDCT for early diagnosis and improved survival. Biomarker testing is decisive in the precision-based management of lung cancer and enables personalized treatment to enhance survival and maintain quality of life. Biomarker analyses focusing on *EGFR*, *ALK*, *ROS1*, PD-L1, and, if feasible, a multigene assay are crucial for precision-based NSCLC management. While broad-panel NGS assays are recommended, challenges such as clinical validation, standardization, costs, and infrastructural requirements must be considered. This consensus provides practical recommendations to overcome barriers, optimize diagnostic strategies, and advance targeted care for improved patient outcomes.

Authors' Contributions

The manuscript has been read and approved by all the authors, the requirements for authorship have been met, and each author believes that the manuscript represents honest work. B.B.: concepts, design, literature search, manuscript preparation, manuscript editing, manuscript review; T.P.: concepts, design, literature search, manuscript preparation, manuscript editing, manuscript review; N.R.: literature search, manuscript editing, manuscript review; V.M.N.: literature search, manuscript editing, manuscript review; B.K.: literature search, manuscript editing, manuscript review; A.S.: literature search, manuscript editing, manuscript review; S.P.: literature search, manuscript editing, manuscript review; J.D.: concepts, design, literature search, manuscript preparation, manuscript editing, manuscript review; S.L.: concepts, design, literature search, manuscript preparation, manuscript editing, manuscript review.

Patient Consent

No patient data was used in this article. Hence patient consent is not applicable for this manuscript.

Conflict of Interest

B.B. received honorarium for lectures/presentations from AstraZeneca, Pfizer, BMS, Intas, DRL, and Alkem. T.P., N.R., V.M.N., B.K., A.S., S.P., J.D., and S.L. declare no conflict of interest.

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Risk Factors of Oral Cancer in India: A Systematic Review and Meta-analysis

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Abstract

Introduction Oral cancer is associated with several well-known risk factors, including the use of betel quid, alcohol consumption, and tobacco smoking. Studies regarding oral cancer risk factors vary based on the different subgroups identified in the Indian context.

Objectives This systematic review, meta-analysis, and meta-regression aggregate data from various studies on oral cavity cancer risk factors in India.

Materials and Methods From September 20 to 30, 2024, we searched for English studies on PubMed, ScienceDirect, and Google Scholar. Two independent reviewers selected studies based on title/abstract and full text, with adjudication by a third author. We utilized the JBI checklist for critical appraisal of case–control studies. The data provided information on participant demographics, cases and controls, evaluated risk factors, and odds ratios. A random effects model produced pooled estimates for each risk factor.

Results Fifteen case–control studies conducted in the Indian population were included in the analysis. Our meta-analysis concludes that any form of tobacco use is the primary risk factor for oral cavity cancer, with risk rising consistently alongside the duration of use. Additionally, daily alcohol consumption significantly increases this risk. Chronic trauma to the oral mucosa also plays a substantial role in the development of oral cavity cancer. Sensitivity analysis and meta-regression indicated that factors such as sample sizes, case–control ratio, and study region had no significant impact. Funnel plots assessing publication bias in studies reporting tobacco smoking and chewing revealed no significant asymmetry, and Egger's test was nonsignificant ($p > 0.05$).

Conclusion There is sufficient evidence for the role of tobacco in both smoking and smokeless forms as a risk factor for oral cavity cancer in India.

Keywords
- risk factors
- oral cancer
- systematic review
- meta-regression
- India

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Introduction

Oral cancer, which affects the lips, *anterior two-thirds* of the tongue, gums, *buccal cavity*, and other areas of the oral cavity, is a significant global health issue. It is the 16th most common cancer worldwide, accounting for over **389,485** new cases and **188,230** deaths annually, particularly in low- and middle-income countries.[1] India has a high prevalence of oral cancer due to various cultural practices, lifestyle choices, and socioeconomic factors. The nation accounts for approximately one-third of the global oral cancer cases as a proportion of its adult population.[2] Despite progress in diagnosis and treatment, the 5-year survival rate remains low, primarily due to *the advanced stage at* detection in rural and urban regions.[3]

Oral cancer is associated with risk factors like chewing betel quid, drinking alcohol, **chewing tobacco,** and smoking tobacco. Globally, using tobacco with areca nut is the most common risk factor.[3] Prolonged use of tobacco and frequent alcohol intake significantly increase the risk.[3] Research shows a higher likelihood of oral cancer among those exposed, with varying odds ratios (ORs) in different populations based on product type, cultural practices, and local laws.[4]

Research on oral cancer risk factors reveals diversity based on specific subgroups considered across different global regions. A thorough review addressing this issue will be beneficial through a critical analysis and summary of the effect measures linking various risk factors to oral cancer from numerous studies.[5] Developing effective preventive strategies and interventions that mitigate the risk of oral cancer necessitates an understanding of the complex nature of these risk factors. This systematic review and meta-analysis aims to compile data from several observational studies regarding oral cavity cancer risk factors conducted in India. Our goal is to furnish public health researchers and policymakers with a valuable estimate of the strength of the relationship between prevalent risk factors and oral cancer in the country.

Materials and Methods

This systematic review and meta-analysis was registered in PROSPERO (CRD42024599556), conducted as per the JBI Methodology for Systematic Reviews of Etiology[6] and reported following the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) 2020 and MOOSE (Meta-analyses Of Observational Studies in Epidemiology) guidelines[7] (►**Supplementary Appendix** [available in the online version only]).

Review Question

Based on current epidemiological evidence, what are the primary risk factors associated with oral cancer in India?

Inclusion Criteria

Participants

This review included adults of any gender over the age of 18 years who reported risk (either a single variable or a group of variables) of oral cavity cancer. Studies were included that reported the risk or equivalent estimates of oral cancer.

Exposure of Interest

Risk factors including but not limited to tobacco use in smoking and smokeless forms (including areca nut and betel quid with tobacco users), duration of usage of tobacco which was categorized as less than or more than 10 years, alcohol consumption which was categorized based on the frequency as daily user or moderate drinker for those who reported occasional usage, chronic oral trauma due to a sharp tooth or ill-fitting denture, diet as measured by frequency of consumption of vegetables and oral cancer. The study did not include studies that primarily examined metabolic parameters (such as obesity), environmental exposures (like sunlight), demographic factors (like age and sex), or genetic predisposition.

Outcomes

The primary outcome of interest was the presence of oral cavity cancer and its association with specific risk factors as measured by ORs or risk ratios (if measured at a specific time-point) or hazard ratios (if measured over time).

Types of Studies

Case–control studies, nested case–control studies, cohort studies, and analytical cross-sectional studies with a comparator arm were included.

Search Strategy

Search strategy was developed and conducted in Medline (PubMed), Science Direct, and Google Scholar databases from inception to September 2024. The keywords included "mouth," "neoplasm," "risk factors," "oral cancer," "carcinoma," "risk predictors," "India" and combined with Boolean operators "*or*" and "*and*." Searches were limited to studies published in English, restricted to India, with no time restrictions. Gray literature, including conference proceedings and dissertations (ShodhGanga, ProQuest), was additionally searched. Included studies underwent backward and forward citation screening to identify any additional studies. Search strategy is described in detail in the ►**Supplementary Material S1** (available in the online version only).

Screening and Identification of Studies

Studies from the search were exported to Rayyan software, underwent duplication, and were screened at the title/abstract and full-text levels by two independent reviewers (M.M. and D.J.), and, where needed, any conflicting decisions were discussed and later included.[8] Studies that reported the association between oral cancer and any

defined risk factors and studies reporting either crude or adjusted ORs or relative risks, along with those providing sufficient data to calculate ORs (e.g., case and control numbers, exposure rates), were included. Those studies reporting incomplete data or insufficient information for statistical pooling, reviews, commentaries, or editorials in languages other than English were excluded. A list of studies excluded from the analysis, with the reason for exclusion, is provided in ►**Supplementary Material S2** (available in the online version only).

Data Extraction

Two independent reviewers (M.M. and D.J.) extracted data from the included studies regarding author names, year of study, location, study design, sample size, age groups, gender, type of cancer, and risk factors along with crude and/or adjusted ORs and corresponding 95% confidence intervals (CIs) according to JBI guidelines. A third reviewer (P.K.) resolved any disagreements.

Critical Appraisal

Critical appraisal of included studies was conducted using JBI checklists for case–control and cohort studies.[6]

Data Synthesis

Studies were grouped based on study design, reported summary measure (crude and adjusted OR or relative risk), and risk factors for oral cancer. Within each group, the risk estimates were pooled using the *metabin* function of meta R package. The estimation of variance within each group was calculated using the DerSimonian-Laird estimator and CIs were determined based on a random effects model.[8]

Sensitivity and Subgroup Analysis

Sensitivity analysis was conducted using the leave-one-out method to detect the source of heterogeneity in the meta-analysis. Sensitivity analysis helps identify whether the pooled estimate was unduly influenced by any single study and improves the stability and robustness of the meta-analysis model.[9] Subgroup analysis was performed based on the region where the study was conducted in India.

Publication Bias

Funnel plots were used to detect any publication bias in the studies by plotting each study's effect size with the sample size of the study and the symmetry of the plot was assessed.

Meta-Regression

To explore the sources of heterogeneity among the included studies, a random effects meta-regression was performed using the "metafor" package in R. The dependent variable was the effect size across studies for smoking and smokeless forms of tobacco and the independent variables were study-level characteristics like sample size, case:control ratio, and the region where the study was conducted. A p-value of <0.05 was considered significant.

Certainty of Evidence

The degree of certainty of evidence was evaluated using the Grading of Recommendations Assessment, Development and Evaluation (GRADE) methodology and conducted separately for each risk factor and outcome association. The GRADE approach evaluates the certainty of evidence based on five domains: risk of bias, inconsistency, indirectness, imprecision, and publication bias.[10] It divides evidence into four levels of certainty: very low, low, moderate, and high. The quality of evidence from the included studies was initially classified as low and subsequently upgraded or downgraded. Inconsistency results in a quality downgrade using a significant difference between studies ($I^2 > 50\%$). Indirectness was considered if there were constraints that limited the result's generalizability. When the 95% CIs for risk estimates are wide or cross a minimally important difference of 10% for outcomes, imprecision was considered. The existence of small-study effects was also considered.

Results

The initial search across all databases and gray literature provided 656 records, of which 42 were duplicates. After a further screening of 614 titles/abstracts, 47 studies that were not conducted in Indian populations or did not address oral cancer were excluded. Ten case–control studies among the 547 reports could be retrieved after undergoing complete screening. Through citation searching, 91 records were found, and 15 reports (1 report not retrieved) were sought for retrieval after duplicates were eliminated; five more reports were included. This resulted in 15 included studies, all with case–control study designs, which included 5,624 cases and 9,151 controls. ►**Supplementary Material S2** (available in the online version only) provides the reasons for exclusion among the studies that underwent full-text screening. ►**Fig. 1** provides the flow chart of the screening process.

►**Table 1** provides the characteristics of the included studies. Only 1 of the 15 studies used a nested case–control design,[11] and the remaining 14 were case–control studies. Eleven studies had oral cavity cancer as the primary outcome variable,[11–21] and 4 of them explicitly identified the outcome as upper aerodigestive tract cancer or oral and oropharyngeal cancer.[22–25] Ten studies were performed in the southern states, and 5 of them,[15,17,22,23,25] were performed in the western region. Tobacco use, either smoking or smokeless, was the most significant risk factor for oral cancer in all of the studies included, with studies examining the type, duration, and quantity of tobacco used.

Smokeless Tobacco Usage and Oral Cancer

Twelve studies with data on smokeless tobacco as a risk factor for oral cancer were included in the meta-analysis. The pooled OR for smokeless tobacco was 5.68 (95% CI: 4.19–7.70; ►**Fig. 2A**). The duration of usage of smokeless form of tobacco less than 10 years had an OR of 1.76 (95% CI:

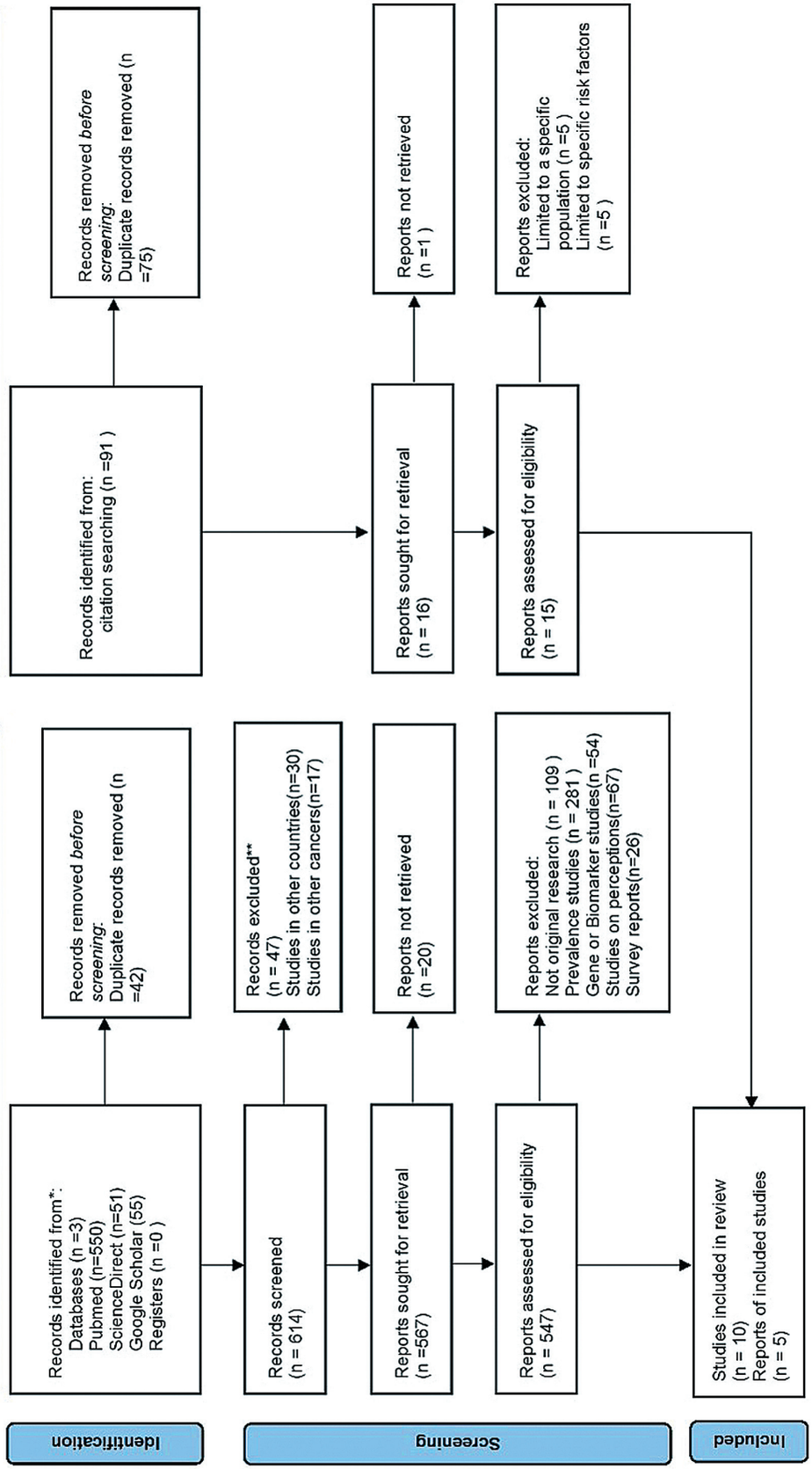
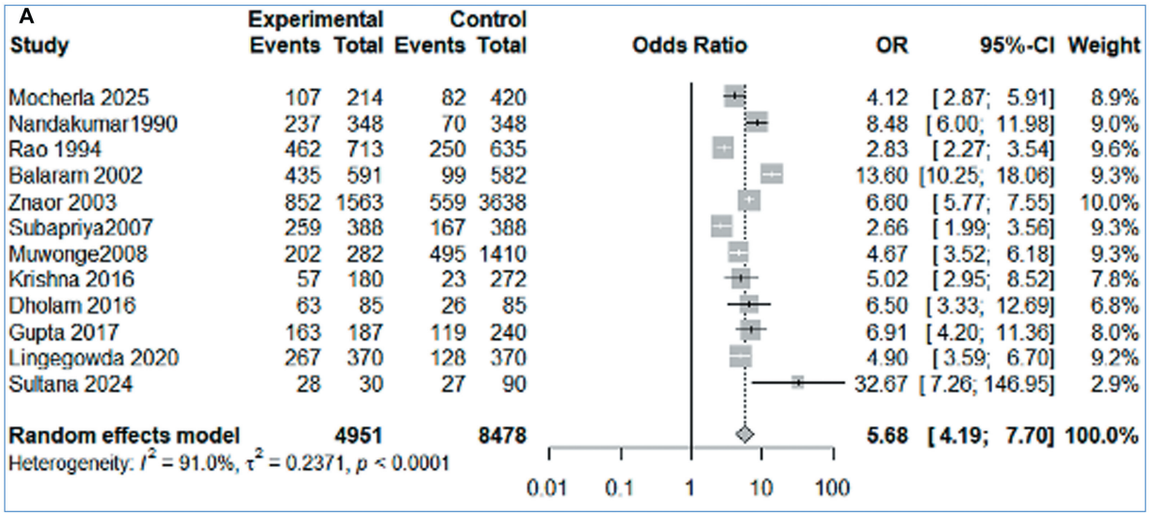


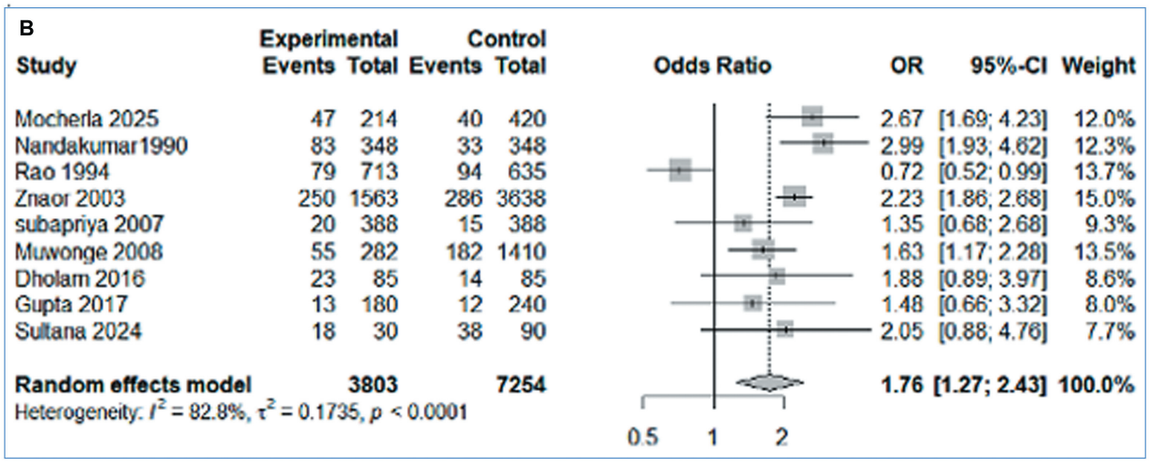
Fig. 1 PRISMA 2020 flowchart.

Table 1 Summary of study characteristics of included studies in the systematic review and meta-analysis

Author, year	State	Region	Sample size		Age range (years)	Gender	Risk factors	Results	Adjustments
			Case	Control					
Nandakumar, 1990	Karnataka	South	348	348	Not given	Both	Smoking and smokeless tobacco (type/number/duration), diet	RR	Not mentioned
Rao, 1994	Maharashtra	West	713	635	18 and above	Males	Smoking and smokeless tobacco, alcohol habits	RR	Age and residence
Wasnik, 1998	Maharashtra	West	123	123	Not given	Both	Smoking and smokeless tobacco (type/number/duration), alcohol, occupation	OR	Not mentioned
Balaram, 2002	Karnataka, Tamil Nadu, Kerala	South	591	582	20–85	Both	Smoking and smokeless tobacco (type/number/years since quitting)	OR	Gender
Znoar, 2003	Tamil Nadu, Kerala	South	1563	3638	25 and above	Males	Smoking and smokeless tobacco (type/number/duration), alcohol (duration/quantity/frequency)	OR	Age, education, center
Subapriya, 2007	Tamil Nadu	South	388	388	30–75	Both	Smoking and smokeless tobacco (type/number/duration), diet, oral hygiene, alcohol (frequency/duration)	OR	Not adjusted
Muwange, 2008	Kerala	South	282	1410	35 and above	Both	Smoking and smokeless tobacco (type/number/duration), alcohol (type/frequency /duration)	OR	Education, religion, habits
Madani, 2012	Maharashtra	West	350	350	18 and above	Both	Smoking and smokeless tobacco (type), diet	OR	Age, gender, education
Dholam, 2016	Maharashtra	West	85	85	18–45	Both	Caries prevalence, oral hygiene, stress, environmental carcinogens, trauma, diet, family history, habits (duration and frequency), placement of quid, BMI, and dental visits	OR	Not mentioned
Krishna, 2016	Karnataka	South	180	272	18 and above	Both	Tobacco habits, diet and oral hygiene behavior	OR	Age and gender
Gupta, 2017	Maharashtra	West	187	240	18 and above	Both	Smoking and smokeless tobacco (type/number/duration), oral hygiene habits, dietary factors, and alcohol drinking	OR	Age and gender
Nirmala, 2019	Karnataka	South	200	200		Both	Smoking (age of initiation/duration/number)	OR	Not adjusted
Lingegowda, 2020	Karnataka	South	370	370	25 and above	Both	Smoking and smokeless tobacco (type/number/duration), alcohol (frequency)	OR	Age and gender
Sultana, 2024	Karnataka	South	30	90	18 and above	Both	Smoking and smokeless tobacco (type/number/duration), diet	OR	Age
Mocherla, 2025	Telangana	South	214	420	18 and above	Both	Smoking and smokeless tobacco (type and duration), alcohol, diet	OR	Age, gender, residence, occupation

Abbreviations: OR, odds ratio; RR, relative risk.





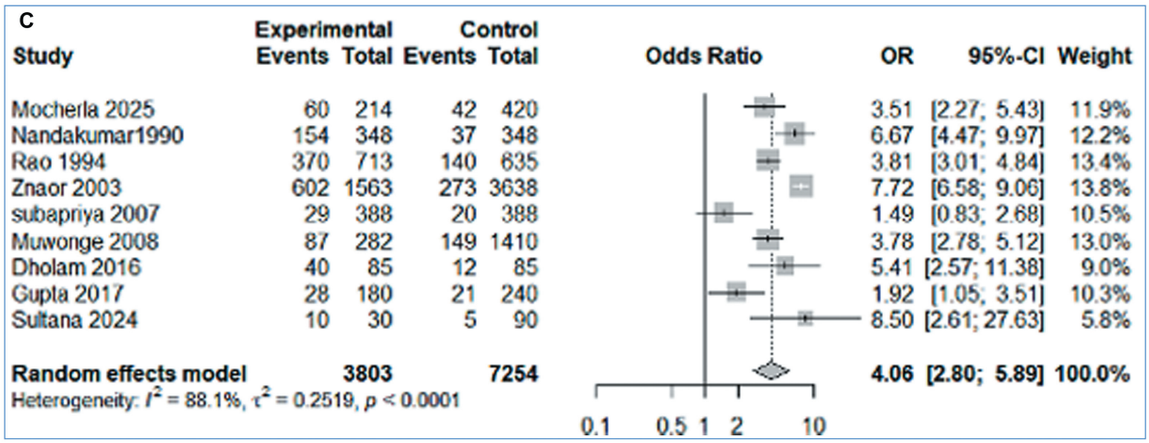
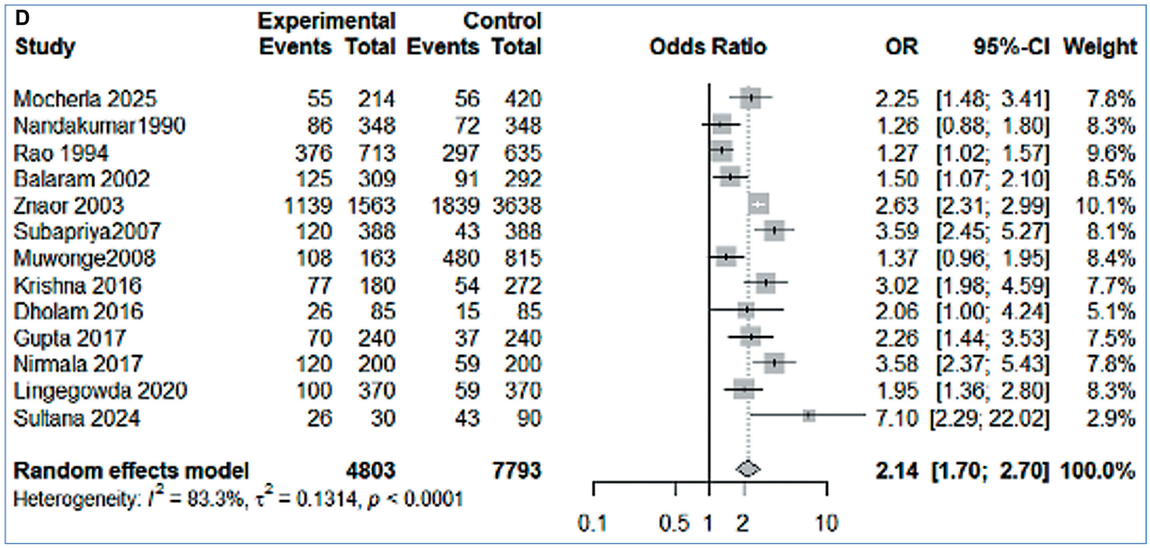
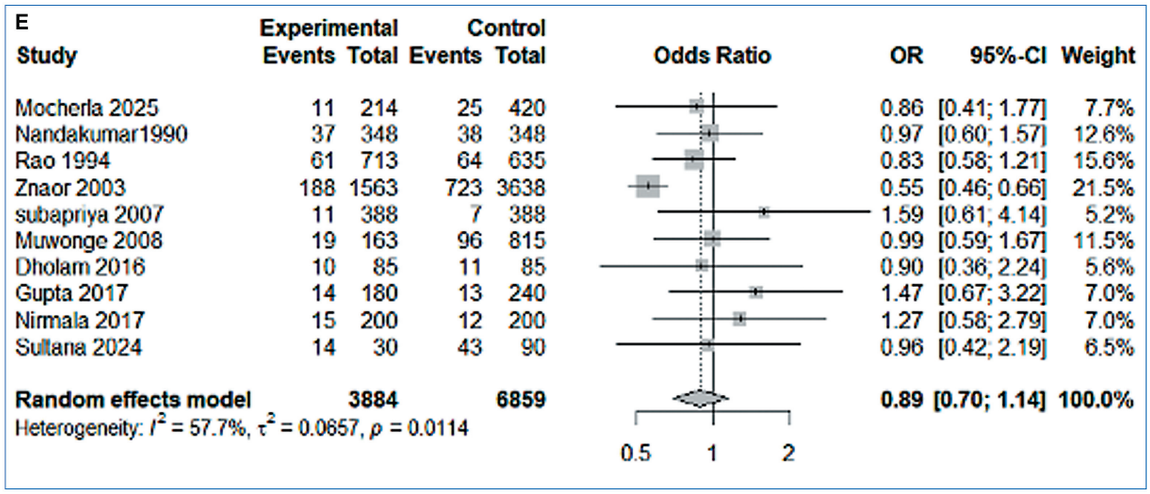


Fig. 2 (A) Tobacco chewing. (B) Smokeless tobacco for less than 10 years. (C) Smokeless tobacco for more than 10 years. (D) Tobacco smoking. (E) Smoking for less than 10 years. (F) Smoking for more than 10 years. (G) Occasional alcohol consumption. (H): Daily alcohol consumption. (I) Consumption of vegetables more than thrice a week. (J) Chronic trauma in the oral cavity.





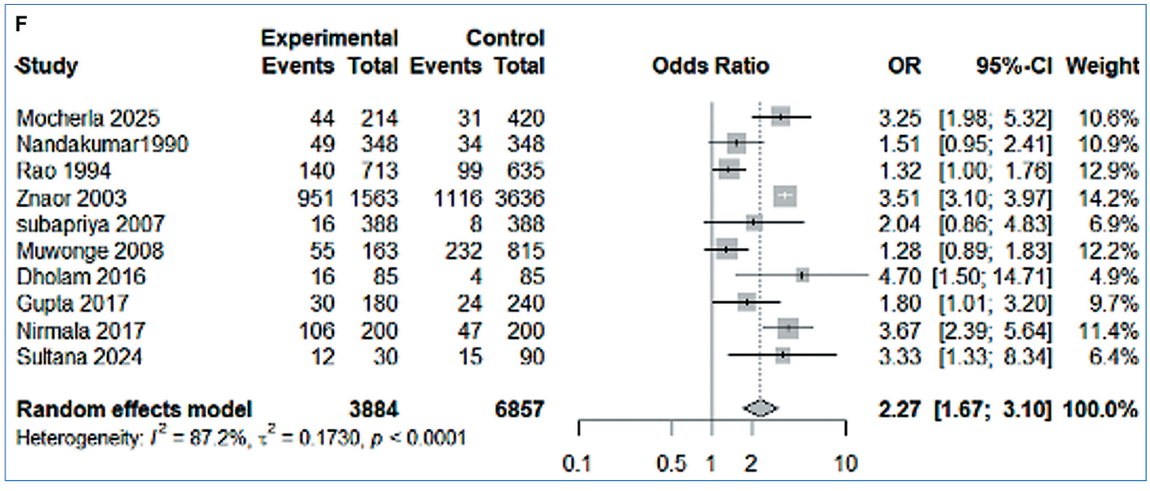


Fig. 2 (Continued)

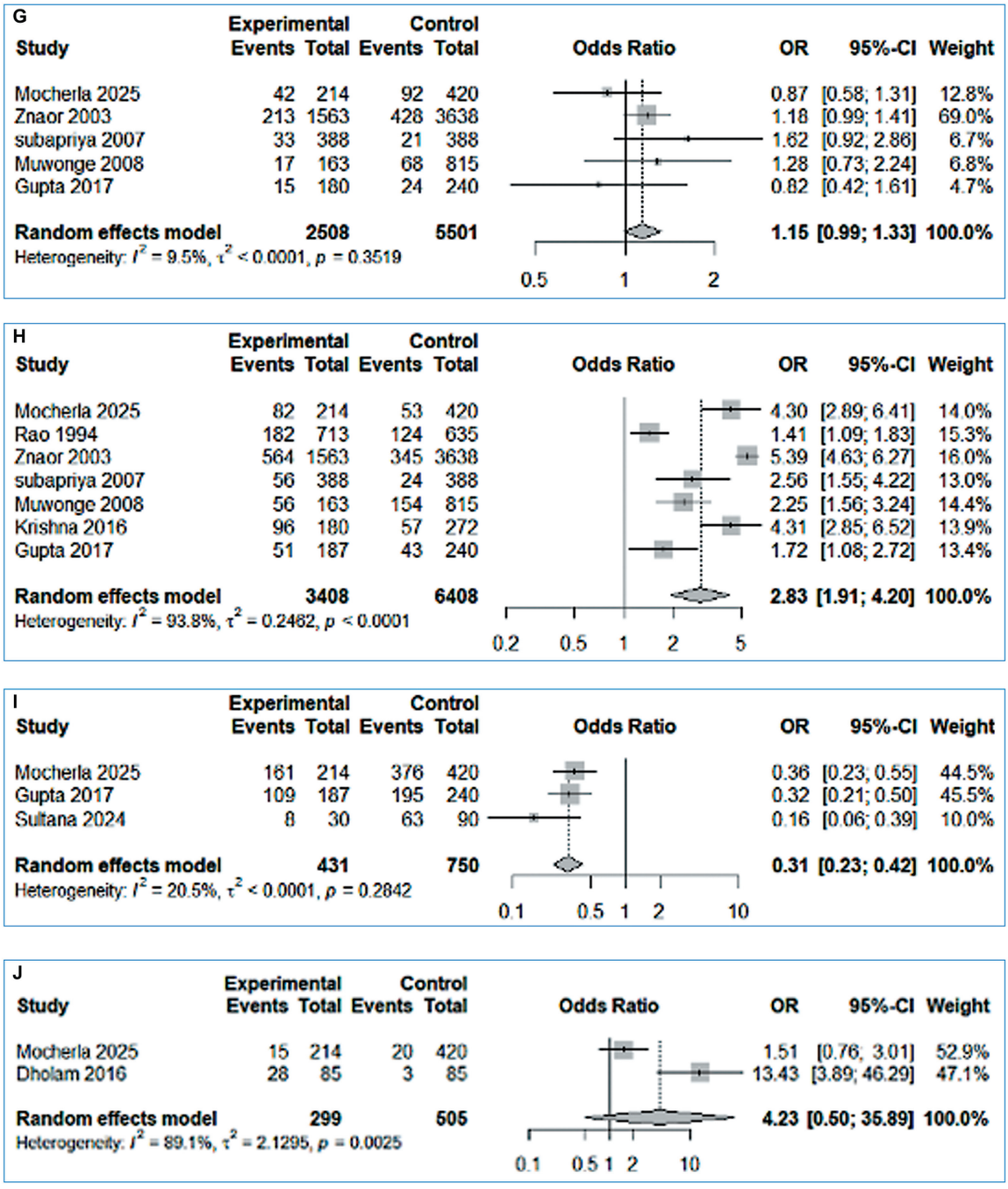


Fig. 2 (Continued)

1.27–2.43; ►**Fig. 2B**), and more than 10 years had an estimate of 4.06 (95% CI: 2.80–5.89; ►**Fig. 2C**).

Smoking Tobacco Usage and Oral Cancer

Thirteen studies included smoking tobacco as a risk factor for oral cancer and were included in the meta-analysis (►**Fig. 2D**). The random effects model generated a pooled OR of 2.11 (95% CI: 1.67–2.65, $p < 0.01$). Duration of usage of smoking tobacco was reported in 11 studies and categorized as less than or more than 10 years (10 studies) and more than 10 years (10 studies). Duration of smoking generated a pooled OR of 0.89 (95% CI: 0.70–1.14; ►**Fig. 2E**) and 2.27 (95% CI: 1.67–3.10; ►**Fig. 2F**), respectively.

Alcohol Consumption and Oral Cancer

Alcohol consumption was identified as a risk factor in seven of the studies. Occasional alcohol consumption was not

significantly associated with oral cancer (►**Fig. 2G**). Daily consumption of alcohol was also a significant risk factor for oral cancer with a pooled estimate of 2.66 (95% CI: 1.92–3.67; ►**Fig. 2H**).

Diet and Oral Cancer

Another significant factor for oral cancer was the frequency with which fruits and vegetables were consumed. Three studies that reported the consumption of vegetables generated an estimate of 0.31 (95% CI: 0.23–0.42; ►**Fig. 2I**).

Chronic Oral Trauma and Oral Cancer

A history of chronic oral trauma and poor oral hygiene was also strongly linked to oral cancer in two studies and the pooled estimate was 1.95 (95% CI = 1.05–3.62; ►**Fig. 2J**).

Subgroup Analysis

The heterogeneity assessed through the random effects model for all the risk factors ranged between 50 and 90%, with a $p < 0.01$. Subgroup analysis based on region revealed no significant variation in the pooled estimates for all risk factors, and heterogeneity ranged from 0 to 90% for all risk factors (►**Table 2**).

Sensitivity Analysis

Sensitivity analysis did not reveal any single study that had a major influence on the pooled estimates (►**Supplementary Material S3** [available in the online version only).

Publication Bias

Funnel plots to assess publication bias in studies reporting tobacco smoking and chewing revealed no major

Table 2 Pooled estimates of risk factors of oral cancer with subgroup analysis based on geographic region

Risk factor		Number of studies	Pooled odds ratio (random effects model)	95% CI	I^2 (%)
Tobacco chewing		14	5.77	4.43–7.53	90
Subgroup	South region	9	6.09	4.21–8.80	91
	West region	5	5.27	3.54–7.85	83
Duration of chewing <10 y		10	1.64	1.19–2.26	82
Subgroup	South region	6	2.13	1.75–2.59	31
	West region	4	1.03	0.62–1.71	58
Duration of chewing > 10 y		10	4.92	3.70–6.52	87
Sub group	South region	6	4.65	3.10–6.97	92
	West region	4	5.37	3.56–8.10	67
Tobacco smoking		15	2.14	1.74–2.62	81
Sub group	South region	10	2.28	1.74–2.98	80
	West region	5	1.86	1.36–2.56	72
Duration of smoking < 10 y		11	0.94	0.73–1.20	61
Sub group	South region	7	0.87	0.64–1.19	62
	West region	4	1.08	0.72–1.60	22
Duration of smoking > 10 y		11	2.35	1.76–3.15	87
Sub group	South region	7	2.71	1.93–3.81	83
	West region	4	1.53	1.17–2.00	38
Occasional alcohol consumption		6	1.09	0.90–1.33	27
Sub group	South region	4	1.16	0.99–1.36	22
	West region	2	0.72	0.43–1.23	00
Daily alcohol consumption		9	2.66	1.92–3.67	92
Sub group	South region	5	3.58	2.55–5.01	84
	West region	4	1.79	1.27–2.53	63
Regular consumption of vegetables		3	0.33	0.22–0.47	45
Chronic trauma to the oral cavity		2	1.95	1.05–3.62	33

Abbreviation: CI, confidence interval.

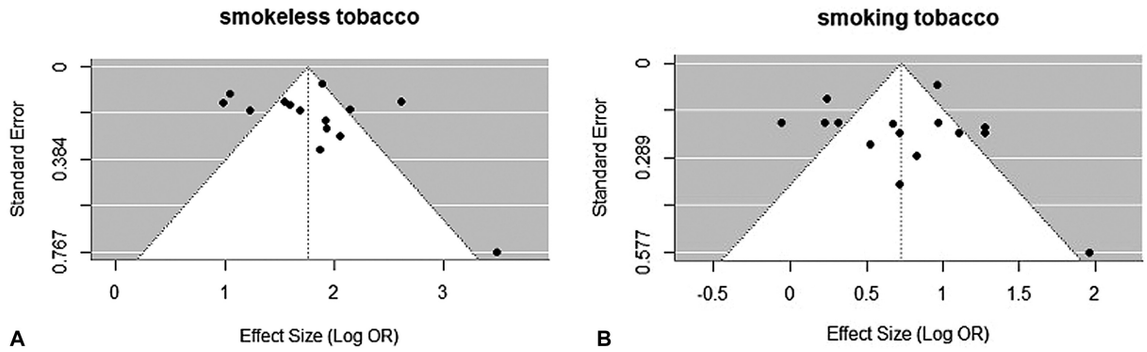


Fig. 3 (A) Smokeless. (B) Smoked.

asymmetry, and Egger's test was nonsignificant ($p > 0.05$; ►**Fig. 3A, B**).

Meta-Regression

Factors such as total sample size, case:control ratio, and the region where the study was conducted had no significant influence ($p > 0.05$) on the pooled estimates (►**Table 3**). Due to the limited number of studies, meta-regressions were not performed for the other factors.

Critical Appraisal

Critical appraisal revealed high evidence for all the studies except two studies[22,24] (►**Supplementary Material S4** [available in the online version only]).

Quality of Evidence

GRADE showed low-quality evidence regarding the association between the risk factors and oral cancer across all studies (►**Supplementary Material S5** [available in the online version only]).

Discussion

In India, awareness of oral cancer and rates of early detection remain low despite high risks. Systematic reviews can provide a comprehensive understanding of *risk factors* and inform targeted interventions for prevention, early detection, and treatment, focusing on these risk factors within the Indian population.[26] Our meta-analysis indicates that any form of tobacco use is the primary risk factor for oral cavity cancer, with risk increasing linearly, correlating with the length of consumption. Additionally, daily alcohol consumption significantly raises this risk, and chronic oral mucosal trauma is another substantial contributor to the development of oral cavity cancer.

Table 3 Meta regression of the association between tobacco chewing/tobacco smoking and sample size, case–control ratio, and the region of conducting the studies

	Estimate	SE	*z*-Value	*p*-Value	CI (lower bound)	CI (upper bound)
Tobacco chewing and sample size						
Intercept	2.1652	0.6572	3.2945	0.0010	0.8771	3.4533[a]
RegionSouth_region	0.2223	−0.5151	−0.4316	0.6661	−1.2319	0.7873
Regionwest_region	−0.1642	0.4315	−0.3806	0.7035	−1.0098	0.6814
case_control_ratio	−0.2716	0.7106	−0.3822	0.7023	−1.6644	1.1212
total_sample_size	−0.0001	0.0002	−0.4865	0.6266	−0.0004	0.0002
Tobacco smoking and sample size						
Intercept	1.3237	0.4607	2.8731	0.0041	0.4207	2.2267[b]
RegionSouth_region	−0.4220	0.3925	−1.0751	0.2823	−1.1912	0.3473
Regionwest_region	−0.1496	0.3026	−0.4943	0.6211	−0.7426	0.4435
Case_control_ratio	−0.5068	0.4532	−1.1182	0.2635	−1.3950	0.3815
Total_sample_size	−0.0001	0.0001	−0.4877	0.6258	−0.0003	0.0002

Abbreviations: CI, confidence interval; SE, standard error.
[a]$p < 0.0001$.
[b]$p < 0.01$.

Tobacco can inhibit several systemic immune functions of the host, alter the epigenetics of oral epithelial cells, and induce OSCC by causing oxidative stress on tissues through its toxic metabolites.[27] Usage of smoking forms of tobacco, irrespective of the subtype, has consistently shown an increased risk of oral cavity cancer.[28] The present analysis yielded a pooled estimate of 2.14 (95% CI = 1.74–2.62). Previous studies from India have reported a similar estimate, ranging from 2.68 (95% CI = 1.90–3.78) in analyses that are not specific to a particular region[26] to 2.2 (95% CI = 0.7–7.0).[29] Duration of smoking less than 10 years did not significantly increase the risk of oral cancer, 0.94 (95% CI = 0.73–1.20) compared with those who smoked for more than 10 years. These findings can be used to encourage people who are in the early stages of developing a tobacco habit to quit as soon as possible.

The current meta-analysis revealed a fivefold increase in the risk of oral cancer among smokeless tobacco users, with the risk increasing consistently with the duration of use. Increased risk of oral cancer with the use of smokeless tobacco has been consistently reported in previous systematic reviews.[30,31] The pooled odds estimates have been reported to be in the range of 3.66 (95% CI: 2.83–4.74) as per global estimates to 7.1 (95% CI: 4.41–11.01) in studies of the southeast Asian region[31] to 5.55 (95% CI: 5.07, 6.07) among studies conducted in India[30,31] which is almost in the same lines as the present analysis, 5.77 (95% CI: 4.43–7.53). The reason for low-risk estimates globally might be due to differences in frequency and intensity of use and variation in the type of smokeless tobacco used. In line with previous studies,[32] a longer duration of usage of smokeless tobacco resulted in a higher estimate, 4.92 (95% CI: 3.70–6.54).

The carcinogenic effects of alcohol consumption on the liver and upper aerodigestive tract are caused by acetaldehyde, the first metabolite of ethanol.[33] Our meta-analysis among the Indian population revealed a pooled estimate of 1.92 (95% CI: 1.44–2.96) for oral cavity cancer among regular alcohol consumers. Though the odds did not reach significant levels ($p > 0.05$) among moderate drinkers (OR = 1.09; 95% CI = 0.90–1.33), the present meta-analysis also confirmed the earlier evidence of daily alcohol consumption as an independent risk factor for oral cavity cancer (OR = 2.66; 95% CI = 1.92–3.67).[16]

Prolonged mucosal damage causes inflammation, which releases chemical mediators like prostaglandins, cytokines, and tumor necrosis factor, results in oxidative stress.[34] This may result in genetic and epigenetic modifications that harm DNA and prevent it from being repaired. Chronic mucosal trauma due to either ill-fitting dentures or a sharp tooth has been proven to be one of the most important risk factors among non-tobacco users. In the present analysis, only two studies reported data related to mucosal trauma resulting in a combined OR of 1.95 (95% CI 1.05–3.62). Singhvi et al reported an OR of 2.62 (95% CI: 2.10–3.25) in a systematic review to assess the role of ill-fitting dentures in the causation of oral cavity cancer.[35] We included chronic trauma from any cause in this analysis, such as a sharp tooth, a broken restoration, or an ill-fitting denture, which may be the reason why our estimates are lower than those of Singhvi et al where ill-fitting dentures, which are the most common cause for chronic mucosal trauma, were considered.

Although this systematic review is thorough, it has several notable limitations. Key risk factors such as family cancer history, stress, and dietary elements like red meat intake related to oral cancer in India could not be evaluated, as none of the collected studies addressed these variables. A detailed evaluation of the quantity and specific forms of tobacco use (e.g., cigarettes, beedis, gutkha, khaini) could not be performed, as most included studies did not consistently report this information. As a result, our analysis was limited to the type and duration of tobacco usage, which may not fully capture dose–response relationships or the differential risks associated with various tobacco products.

Additionally, HPV's role was not examined, as previous research has mainly associated it with oropharyngeal cancer, and not with oral cavity cancer. The high levels of heterogeneity among the studies included in the present meta-analysis should also be considered when interpreting the pooled estimates. Nevertheless, even the lowest effect estimates among the individual studies are more significant than 1, suggesting a causal relationship between tobacco usage and oral cancer. Regional variations in product composition and population characteristics may cause the wide variation in effect estimates across individual studies.[30] One must bear these limitations in mind while analyzing the findings. Exposure data are frequently inaccurate when categorized either qualitatively by product type or quantitatively by frequency of consumption. The bias was reduced by grouping data into broad categories, such as ever or never using tobacco products, and having used them for less than or more than 10 years. Based on the meta-analysis results, the GRADE assessment of included studies showed low confidence. Future research could concentrate on high-quality studies to improve the quality of the evidence.

Numerous studies have investigated the impact of both smoking and smokeless tobacco, as well as alcohol consumption, on oral cancer within the Indian context. Nevertheless, the interplay between tobacco and alcohol requires more in-depth examination. Additionally, chronic trauma to the oral cavity and its potential role in triggering carcinogenesis represent another area worthy of further research. To effectively address the rising incidence of oral cancer, a comprehensive strategy is essential. This necessitates a collaborative effort among lawmakers, healthcare providers, and the public to raise awareness, change behaviors, and implement preventive measures.

Conclusion

This meta-analysis provides sufficient evidence for the role of tobacco in both smoking and smokeless forms as a

risk factor for oral cavity cancer, which includes the tobacco-related and the non-tobacco-related factors in Indian populations. The results from the meta-analysis emphasize how long-term tobacco use and chronic oral trauma are significant and underexplored risk factors for cancer of the oral cavity in the country. By evaluating the degree of evidence certainty, the GRADE approach strengthens the methodology. These results lend support to future studies that aim to enhance risk prediction algorithms and develop targeted measures for early identification and prevention.

Data Availability Statement
Available as supplementary files.

Patient Consent
Not applicable.

PROSPERO Registration
CRD42024599556.

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Conflict of Interest
None declared.

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Navigating the Genetic Frontier: Establishing Genetic Clinics for Comprehensive Hereditary Cancer Management in Resource-Limited Settings

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Abstract

This review article explores the vital role of genetic clinics in the management of hereditary cancers, emphasizing their significance in early detection, tailored treatment, and family screening. It presents analysis of the prevalence of hereditary cancers in India, including breast, ovarian, prostate, colon, and kidney cancers, stressing the necessity for effective genetic testing and counseling services.

The manuscript synthesizes available prevalence data, outlines key components and challenges of clinic establishment, and provides strategies for improving public acceptance, access, and workforce capacity. It also focuses on importance of early detection, family screening, and targeted therapies for patients with inherited genetic mutation.

In conclusion, the article emphasizes the need for a patient-centered approach, increased access to genetic clinics, exploration of future genomics and technology advancements, and the evaluation of genetic clinics' impact on patient outcomes. By addressing challenges and capitalizing on opportunities, genetic clinics can continue to improve patients' and their families' lives, ultimately contributing to the battle against hereditary cancers.

Keywords

- genetic clinics
- hereditary cancers
- genetic testing and counselling
- cancer prevention and management
- awareness and acceptance

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Introduction

The growing burden of hereditary cancers in India has highlighted the need for a comprehensive approach to their management, detection, and prevention. Hereditary cancers result from inherited gene mutations that significantly increase an individual's risk of developing cancer. These mutations are responsible for a considerable proportion of cancer cases in India, including breast (5–10%), ovarian (15–20%), prostate (5–9%), colon (2–6%), and kidney (5–8%) cancers.[1] Despite the substantial impact of hereditary cancers, limited work has been done to address this issue in India.

The lack of awareness and knowledge about hereditary cancers in both the general population and the health care community has led to inadequate genetic testing and counseling services.[2] This shortfall contributes to a delayed diagnosis, less effective treatments, and missed opportunities for early intervention and prevention. Furthermore, the limited availability of genetic clinics, trained cancer genetic counsellors, and the high cost of genetic testing services have made access to these essential services challenging for many individuals and families.[3]

In recent years, however, there has been a growing recognition of the importance of genetic clinics in addressing the burden of hereditary cancers in India. Genetic clinics play a crucial role in providing comprehensive care by offering genetic testing and counseling services that enable early detection, personalized treatment, and family screening. Establishing genetic clinics in new cancer centers can significantly improve the management of hereditary cancers and enhance the overall quality of care for patients and their families.[4]

By shedding light on the current state of hereditary cancer management in India and outlining strategies to improve access to genetic clinics, this article aims to contribute to the ongoing efforts to combat hereditary cancers and improve patient outcomes. ►**Table 1** provides an overview of the available data on hereditary cancers in India.

Importance of Genetics Clinic

Genetics clinics play a vital role in the management of hereditary cancers. They provide numerous benefits, including:

Early Detection and Prevention

Genetic testing enables early identification of individuals at risk for hereditary cancers. It allows for the implementation of appropriate screening programs and prophylactic interventions, reducing morbidity and mortality rates.

Therapeutic Benefit

Genetic testing can identify specific mutations that may be targeted by personalized therapies. For example, patients with BRCA1/2 mutations may respond well to poly (ADP-ribose) polymerase (PARP) inhibitors, improving their prognosis.[5]

Family Screening

Screening relatives of patients with hereditary cancers can help identify at-risk family members, enabling early interventions and better management of the disease.

Steps in Setting Up a Genetic Clinic in a New Cancer Centre

Establishing a genetic clinic involves several crucial steps (►**Table 2**):

Define the Scope

Identify the types of services offered, such as genetic counseling, testing, and personalized treatment planning.

Create a Multidisciplinary Team

Assemble a team of experts, including trained cancer genetic counselors, oncologists, surgeons, and laboratory personnel.

Develop Standard Operating Procedures

Develop and implement standard operating procedures for genetic testing, counseling, and management of patients with hereditary cancers.

Obtain Necessary Equipment and Resources

Acquire the necessary equipment for genetic testing, such as next-generation sequencing platforms, and ensure proper training of laboratory staff.

Establish Partnerships

Collaborate with other cancer centers, academic institutions, and research organizations to share resources and knowledge.

Table 1 Prevalence of hereditary cancers in India

Cancer type	Percentage of hereditary cases	Linked genes	Suggested solutions for early detection
Breast	5–10%	BRCA1, BRCA2	Genetic testing, mammography, MRI
Ovarian	15–20%	BRCA1, BRCA2	Genetic testing, ultrasound, CA-125 test
Prostate	5–9%	HOXB13	Genetic testing, PSA test, DRE
Colon	2–6%	APC, MUTYH	Genetic testing, colonoscopy, FOBT
Kidney	5–8%	VHL, MET	Genetic testing, CT scan, MRI

Abbreviations: CT, computed tomography; DRE, digital rectal exam; FOBT, fecal occult blood test; MRI, magnetic resonance imaging; PSA, prostate-specific antigen.

Table 2 Steps in setting up a genetic clinic

Step #	Description	More details
1	Define the scope	Identify target population, services offered
2	Create a multidisciplinary team	Include genetic counselors, physicians, etc.
3	Develop standard operating procedures	Create guidelines for genetic testing, follow-up
4	Acquire necessary resources	Secure funding, equipment, and space
5	Establish partnerships	Collaborate with hospitals, research institutions

Table 3 Challenges associated with genetic clinics

Challenge #	Description	Suggested solutions
1	Limited awareness and acceptance	Educational programs, community involvement
2	Financial constraints	Seek funding, collaborate with stakeholders
3	Ethical concerns	Develop ethical guidelines, informed consent

Challenges Associated with Setting Up a Genetic Clinic

Several challenges need to be addressed when establishing a genetic clinic (►**Table 3**):

Limited Awareness and Acceptance

In many communities, there may be limited awareness and acceptance of genetic testing. It is essential to develop strategies to educate patients and the public about its benefits.

Financial Constraints

One of the biggest barriers to the uptake of genetic testing is its cost. Many patients cannot afford the initial genetic test, and if found to be mutation carriers (previvors), they often face the additional financial burden of regular, lifelong cancer screening and preventive interventions. These may include annual magnetic resonance imaging, mammograms, colonoscopies, or even risk-reducing surgeries—all of which are expensive and rarely covered by insurance in low- and middle-income countries (LMICs). This creates a significant equity gap, where only a small fraction of the population can access the benefits of personalized cancer prevention.

To overcome this, public cancer centers must advocate for state-funded testing programs for high-risk groups, collaborate with nonprofit foundations to sponsor testing and screening, and push for integration of genetic services into national health insurance schemes. Examples from other LMICs suggest that cost-sharing models, means-based subsidies, or donor-funded screening programs can improve accessibility. Public hospitals can collaborate with government schemes (e.g., Ayushman Bharat), nongovernmental organizations, and insurance companies to subsidize costs.

Shortage of Skilled Personnel

There may be a limited number of trained cancer genetic counselors and other specialists available to run a genetic clinic. Training programs and partnerships with academic institutions can help address these challenges.

Ethical Aspects of Genetic Clinics

Ethical considerations are an integral part of genetic clinic operations:

Informed Consent

Obtaining informed consent from patients before genetic testing is essential to ensure they understand the implications, risks, and benefits.

Confidentiality and Privacy

Protecting patients' genetic information is critical to maintain their privacy and prevent potential discrimination. Implementing strict data security measures is necessary.

Strategies for Improving Acceptance of Genetic Testing

To enhance the acceptance of genetic testing in society, various methods can be employed:

Public Awareness Campaigns

Utilize mass media, social media, and community outreach programs to raise awareness about the benefits of genetic testing.

Collaboration with Primary Care Providers

Collaborate with primary care providers to identify at-risk patients and refer them to genetic clinics for further evaluation and testing.

Financial Assistance Programs

Establish programs to help patients cover the cost of genetic testing and related services.

Enhancing Access to Genetic Clinics

Improving access to genetic clinics is vital for ensuring that a broader population can benefit from their services. Some approaches to achieve this goal include the following:

Table 4 Benefits of genetic testing

Benefit #	Description	Implications for patients and families
1	Therapeutic benefits	Tailored treatments based on genetic profile
2	Early detection	Timely interventions, better prognosis
3	Family screening	Identify at-risk family members, prevention

Telemedicine and Remote Genetic Counseling

Leveraging telemedicine and remote genetic counseling can help reach individuals in rural or remote areas where access to genetic clinics may be limited. This approach may also be useful in providing services to patients with mobility or transportation challenges.

Mobile Genetic Clinics

Establishing mobile genetic clinics that can travel to underserved areas can increase access to genetic testing and counseling for individuals who might otherwise not receive these services.

Collaboration with Community Health Centers

Partnering with community health centers can help to identify individuals at risk for hereditary cancers and facilitate appropriate referrals to genetic clinics.

Discussion

Genetic clinics are essential for the management of hereditary cancers, offering numerous benefits such as early detection, therapeutic benefits, and family screening (►**Table 4**).

The process of setting up a genetic clinic within a cancer center can be met with several challenges.[4,6] One such challenge is ensuring access to high-quality genetic testing services that provide accurate and reliable results. Moreover, the lack of well-established guidelines for genetic testing and management of hereditary cancer syndromes in India further complicates the process.[7]

In addition to technical challenges, financial constraints can pose significant barriers to establishing a genetic clinic. The high cost of genetic testing and limited funding opportunities for research and service development contribute to these challenges. Additionally, there is a need to train health care professionals in the field of cancer genetics, which requires resources and infrastructure.

Implementing multidisciplinary care models that include oncologists, cancer genetic counselors, and other health care professionals can improve patient outcomes.[8] However, a shortage of trained genetic counselors and other professionals can hinder the formation of these multidisciplinary teams. Moreover, the lack of awareness about the importance of genetic counseling and testing among health care professionals further exacerbates the problem.

Ethical considerations also play a critical role in the establishment of genetic clinics. Issues such as informed consent, patient confidentiality, and the disclosure of genetic information to family members must be addressed. Genetic counseling should be sensitive to cultural and religious beliefs, as well as the psychological impact of genetic testing on patients and their families.[9]

Public awareness of the benefits of genetic testing and counseling is vital for its acceptance and utilization. Social media can be an effective tool for disseminating information and promoting awareness about the importance of genetic testing. Studies have shown that social media interventions can improve patient well-being and facilitate social support. Furthermore, social media can be used to engage with diverse populations and promote health behavior changes.[10]

Mass media campaigns have also been proven effective in promoting health behavior change and reducing the incidence of cancer. Tailored interventions targeting specific populations can further improve the efficacy of these campaigns. By leveraging new technologies, health care providers can enhance the prevention and management of chronic conditions, including hereditary cancers.[11]

Outreach and integration programs can promote family communication and participation in genetic counseling and testing among low-income and underserved populations. These programs can be especially beneficial for minority groups who may have limited access to genetic services due to cultural, linguistic, or financial barriers.[2] By addressing these challenges and fostering an environment that encourages genetic testing and counseling, cancer centers can harness the potential of genetic clinics to improve cancer prevention and management (►**Table 5**).

By integrating culturally sensitive approaches and addressing disparities, health care professionals can ensure that a diverse population can benefit from the advancements in genetic testing and counselling.[12]

Table 5 Strategies for enhancing genetic testing acceptance

Strategy #	Description	More details
1	Educational programs	Public campaigns, health care provider education
2	Community involvement	Health fairs, workshops, patient advocacy
3	Addressing cultural sensitivities	Develop culturally appropriate resources, counseling

Limitations

This review has several limitations. First, much of the data on the prevalence and uptake of genetic testing in India and other LMICs remain sparse or institution-specific, limiting generalizability. Second, the recommendations provided may not be uniformly applicable due to variability in health care infrastructure, resource availability, and sociocultural dynamics across regions. Third, while international practices and models are referenced, contextualizing them to Indian or LMIC settings may require further field validation. Finally, a lack of patient-centered outcome data hinders the ability to fully quantify the impact of genetic clinics on long-term health outcomes and cost savings.

Conclusion

Genetic clinics are pivotal in the era of personalized medicine, particularly for hereditary cancer prevention and management. Their role in early detection, therapeutic decision-making, and cascade testing has far-reaching implications for reducing cancer incidence and mortality. However, the success of such clinics in LMICs hinges on overcoming structural, economic, and sociocultural barriers. By investing in infrastructure, workforce training, community engagement, and inclusive policy development, LMICs can unlock the full potential of genetic clinics. Moving forward, tailored implementation strategies, continuous evaluation, and stakeholder collaboration will be essential in ensuring sustainable and equitable access to these critical services.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study

Patient Consent

No patient data was collected as informed consent was not required.

Conflict of Interest

None declared.

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Pattern of Expression of CDX2 in Colorectal Cancer and its Role in Prognosis: An Ambispective Observational Study

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Abstract

Introduction Caudal-type homeobox 2 (CDX2), a nuclear protein, is essential for the proliferation and development of intestinal epithelial cells and is frequently down-regulated during tumorigenesis. CDX2 inhibits cell growth as well as stimulates differentiation by activating intestinal specific genes, thus lack of CDX2 favors tumor growth and aggressiveness.

Objectives We aimed to evaluate the pattern of CDX2 expression in all stages of colorectal cancer (CRC) and study its association with baseline characteristics and prognosis.

Materials and Methods Study was conducted as an ambispective observational study, enrolling cases of CRC retrospectively from January 2014 to July 2016 (30 months), and prospectively during next 18-month period till January 2018. We performed CDX2 staining by immunohistochemistry on the available biopsy blocks of CRC patients during the study period. Total 286 patients were registered during the study period, of which only 110 biopsy blocks were available for staining. CDX2 scoring was done by a semiquantitative method on whole tissue section for the intensity and percentage of the cells showing positivity. Correlation of CDX2 expression was done with baseline clinical and histopathologic characteristics, and survival.

Results Of 110 patients, 77 (70%) constituted colon cancer and 33 (30%) were rectal cancer. The median age was 54.2 years, 62 (56.4%) being male and 48 (43.6%) female with male-to-female ratio 1.3:1. In the study cohort, 33 (30%) patients had stage II disease, 30 (27.3%) stage III, and 47 (42.7%) were stage IV. Seventy-three (66.4%) were positive for CDX2 and 37 (33.4%) were negative. Loss of CDX2 expression was significantly associated with advanced stage, rectal site, poor grade of differentiation, and presence of lymphovascular invasion (LVSI). With median follow-up of 16 months, progression-free survival (PFS) at 2 years was 30% for CDX2 negative patients compared

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with 67% for CDX2 positive ($p = 0.009$), while overall survival (OS) at 2 years was 46% for CDX2 negative versus 77% for positive patients ($p = 0.01$).

Conclusion Loss of CDX2 expression is associated with advanced stage, higher tumor grade, presence of LVSI, and worse PFS and OS and thereby functions as a poor prognostic factor in CRC.

Introduction

Colorectal cancer (CRC) is one of the common cancers diagnosed worldwide and is considered as the second among the leading causes of cancer deaths.[1] The incidence and mortality from CRC is more in developed countries.[2] Approximately 20% of the patients with CRC presents with distant metastasis at diagnosis.[3]

Numerous prognostic markers, related to the pathogenesis of CRC have been studied to predict treatment outcome.[4] Several of these biomarkers are not reproducible and are not robust enough to find place in clinics. A few of these markers have come to clinical practice to help in knowing the prognosis and/or predicting response to particular therapy, either fluorouracil (5FU)/oxaliplatin-based chemotherapy or biological therapy. There is ongoing research to find more novel prognostic or predictive biomarkers or panel of markers at molecular levels to better stratify patients and determine systemic treatment based upon the risk groupings.[5]

Among the novel biomarkers, caudal-type homeobox 2 (CDX2) a nuclear transcription factor, has been explored to predict the survival outcomes in patients with CRC.[6,7] CDX2 is an intestinal factor, encoded by CDX2 gene which is important for homeostasis and development of the intestinal epithelium.[8] CDX2 inhibits cell growth as well as stimulates differentiation by activating intestinal specific genes.[9,10] Loss of CDX2 expression is seen in almost 30% of human CRC and is associated with high tumor grade.[11] CDX2 expression can be identified both by gene expressing profile and immunohistochemistry (IHC).[12] A recent study has validated the prognostic and predictive utility of CDX2 in a large cohort of early-stage colon cancer.[13] Paucity of data on the role of CDX2 expression in advanced colon cancer, in rectal cancer, and in Indian population had surged us to undertake this work.

In our present study, we aimed to characterize the expression of CDX2 by IHC in all stages of colon as well as rectal cancers and to identify its association with various baseline clinical and histopathological parameters. Overall response rates (ORRs) and survival outcomes were evaluated in relation to CDX2 expression within the study cohort.

Material and Methods

Patient Recruitment and Data Collection

The study was conducted as an ambispective observational study. Cases were enrolled into the study both retrospectively (from January 2014 to July 2016; 30 months) and prospectively from July 2016 till January 2018 (18 months). Last case enrolled was followed up for a minimum of 6 months until the day of study analysis in July 2018. Cases enrolled into this study were identified from the central computerized database in the department of medical oncology at a tertiary cancer care center in South India. All newly diagnosed cases, age $\geq$ 18 years, any stage (early or advanced), with histopathologically proven diagnosis of CRC were considered for inclusion. Cases were excluded from the study if sufficient tissue material (either a biopsy or postoperative tissue blocks) was not available to perform IHC. Convenience sampling method was used for study enrollment and all consecutive cases meeting the inclusion/exclusion criteria registered during the study period were included into the study. Among 286 patients of CRC registered in the participating department during the study duration of January 2014 to January 2018, 110 cases met the inclusion criteria and were enrolled for analysis. Others ($n = 176$) were excluded due to unavailability of biopsy block or unstained slides.

Baseline clinical, histopathological, treatment, and follow-up details were collected from the medical file records. Baseline biopsies were reviewed for reconfirmation of pathological diagnosis for all eligible patients. Following outcome measures were considered for the study: primary measures: (1) percentage of CDX2 expression in the study cohort, (2) response rate in patients with advanced CRC, and (3) progression-free survival (PFS), and secondary outcome measures: (1) overall survival (OS).

Immunohistochemical Staining and Analysis of CDX2

Whole tissue section from the archived paraffin blocks were used and IHC staining for CDX2 was performed by using monoclonal mouse anti-human CDX2 (clone 88) (DAKO, USA) antibodies. Analysis of these was done by a semiquantitative method using a light microscope with a 40$\times$ objective at a total magnification of $\times$400. Area of interest (tumor cell regions avoiding areas containing fibrosis or necrosis) on stained slides was identified and representative 20 high power fields (HPF) were selected for scoring of the cell. The relative percentage of CDX2 positive cells in relation to overall cellularity was calculated for each HPF and the final score for an individual patient was taken as an average of the 20 HPF.

CDX2 Scoring

CDX2 was considered positive only for nuclear staining. Any cytoplasmic positivity was considered artifactual. Positivity of any intensity was considered as positive.[8] Normal colonic

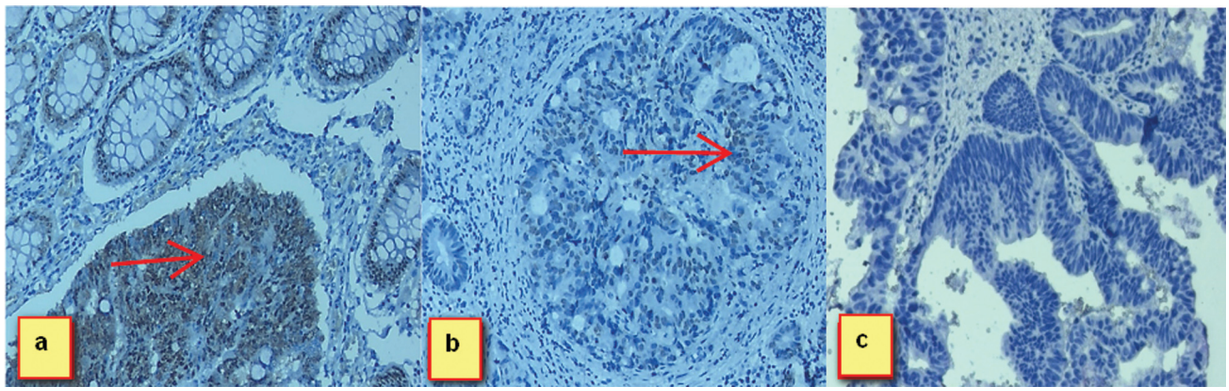

Fig. 1 (A) Immunohistochemistry (IHC) stain, 40 ×. Tumor cells show strong diffuse caudal-type homeobox 2 (CDX2) nuclear positivity along with normal intestinal mucosa. (B) IHC stain, 40 ×. Focal weak nuclear CDX2 expression in tumor cells. (C) IHC stain, 40 ×. Tumor cells are negative for CDX2 expression.

mucosal tissue was used as control. ►**Fig. 1** demonstrates positive and negative staining of CDX2.

CDX2 scoring was done in semiquantitative method for the intensity and percentage of the cells showing positivity. Scoring was done according to the following pattern described by Bayrak et al[14]: "less than 5% of tumor cells were given score of 0, 5 to 25% of tumor cells positive for CDX2 considered as 1+, 26 to 50% of tumor cells positive for CDX2 as 2+, 51 to 75% of tumor cells positive for CDX2 were taken 3+, and greater than 75% of tumor cells positive for CDX2 as 4+." Additionally, positivity for CDX2 was also classified in the following subgroups: focal positivity if less than 50% of tumor cells were positive and diffuse positivity staining in more than 50% of the tumor cells.[14]

Statistical Analysis

Descriptive statistics was used for baseline characteristics, disease factors, laboratory, histopathological, treatment details, and CDX2 expression. Correlation of CDX2 expression with baseline clinical and histopathologic characteristics was done using chi-square test/Fisher's exact test for categorical data. Response rate with respect to CDX2 was calculated using chi-square test. Kaplan–Meier method was used for survival estimation and log-rank test was used for comparison. Median follow-up time for the cohort was calculated using the reverse Kaplan method for potential follow-up. Cox proportional hazards method was used to evaluate significance of CDX2 expression in predicting survival outcomes, while adjusting for other parameters with a significance level below 0.25 at a univariate level. Two-sided *p*-value of less than 0.05 was considered statistically significant. All statistical analysis was done using SPSS version 20.0 and data was censored on June 30, 2018 for survival analysis.

Endpoint definitions: PFS was defined as the time from initial diagnosis to progression at any time, relapse from complete response (CR), initiation of new previously unplanned treatment, or death from any cause. OS was defined as the time from date of registration till date of last follow-up or death from any cause. ORR for advanced stage CRC was defined as partial response or CR to chemotherapy according to response evaluation criteria in solid tumors (RECIST) criteria 1.1.[15]

Ethics: The study was approved by the institutional research and ethics committee (vide letter no. JIP/IEC/SC/2016/29/897 dated 12/07/2016). Informed consent was obtained from cases enrolled prospectively while a waiver of consent was granted for the retrospective cases. The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1964, as revised in 2013.

Results

A total of 110 cases were included into the study for analysis. Summary of the main results is described below.

Baseline Characteristics

The median age of the study cohort was 51 years (range 21–79 years); 62 (56.4%) were male and 48 (43.6%) were female. Among 110 patients, 77 (70%) had colon cancer; 38 (34.5%) patients had right-sided tumor and 39 (35.5%) had left-sided colon involvement, and 33 (30%) had rectal cancer (►**Table 1**). A significant proportion of patients, 42.7% ($n = 47$) had stage IV CRC, while 33 patients (30%) had stage II disease and 30 (27.3%) had stage III disease. Stage II patients had various overlapping high-risk features, mainly inadequate lymph node dissection in 20 (60.6%), and presence of lymphovascular invasion (LVSI) in 7 (21.2%). Serum carcinoembryonic antigen (CEA) levels were available for 78 patients. Median CEA level was 5.5 ng/mL with interquartile range (IQR) of 0.7 to 24.7 ng/mL. Sixty (72.3%) patients had elevated serum CEA levels while 23 (27.7%) had normal serum CEA levels.

Histopathological Details

Of the 110 patients in the analysis, 9 (8.2%) had mucinous histology. Based on grade of differentiation, grade I was the most common, seen in 62 (56.4%) patients, 34 (30.9%) had grade II, and 5 (4.5%) had grade III tumors. Out of 80 patients who underwent definite surgery, 38 (47.5%) had adequate lymph node dissection, 36 (45%) had inadequate lymph node dissection, and no information was available for 6 (7.5%) patients. Among 74 patients out of 80 whose lymph node

Table 1 Baseline demographic characteristics

	Features	Patients		Total $n = 110$
		Colon (77)	Rectum (33)	
1.	Age (median age): 51.47 y (21–79 y)			
	(a) $\leq$ 30 y	7	3	10 (9.1%)
	(b) 31–60 y	47	20	67 (60.9%)
	(c) $>$ 60 y	23	10	33 (30%)
2.	Gender			
	(a) Male	42	20	62 (56.4%)
	(b) Female	35	13	48 (43.6%)
3.	Performance status			
	(a) 0–1	52	26	78 (70.9%)
	(b) 2	17	7	24 (21.8%)
	(c) 3	8	0	8 (7.3%)
4.	Site of disease			
	(a) Right colon	38	–	38 (34.5%)
	(b) Left colon	39	–	39 (35.5%)
	(c) Rectum	–	33	33 (30%)
5.	Stage of disease			
	(a) Stage II	28	5	33 (30%)
	(b) Stage III	21	9	30 (27.3%)
	(c) Stage IV	28	19	47 (42.7%)
6.	Symptom duration (median): 3 mo (0–24 mo)			
	(a) $<$ 1 mo	13	2	15 (22.7%)
	(b) 1–6 mo	50	25	75 (68.2%)
	(c) $>$ 6 mo	14	6	20 (18.1%)
7.	Symptoms[a]			
	(a) Obstruction and perforation	12	1	13 (11.2%)
	(b) Abdominal pain/distension	57	15	72 (62.1%)
	(c) Vomiting	23	1	24 (20.7%)
	(d) Bleeding PR	15	23	38 (32.8%)
	(e) Altered bowel habit	10	8	18 (15.5%)
	(f) Decreased appetite/weight	3	1	4 (3.4%)

Abbreviation: PR, partial response.
[a]Overlapping symptoms may be present.

status was available, 42 (56%) were positive and 32 (44%) had negative lymph node involvement. Positive LVSI was seen in 35% patients and 3.7% had unknown LVSI status. Molecular studies were available for very few patients. KRAS/NRAS testing was done in 4 patients, 3 were wild-type and one was mutated. Six patients were tested by IHC for mismatch repair (MMR), and all were MMR proficient.

CDX2 Expression

All the 110 patients of CRC were evaluated for CDX2 marker, a nuclear protein, by IHC with anti-CDX2 clone 88 antibodies. With the use of scoring system as described in methodology, 73 (66.4%) patients were positive for CDX2 and 37 (33.6%) were negative as shown in ►**Table 2**. Median CDX2 positivity was 26.5% (IQR 1–70). In 37 patients who had negative CDX2 expression ($<$ 5% of tumor cells positive), 22 patients had a score of 0, that is, no CDX2 expression at all and 15 patients had CDX2 score of 1 to 4%.

Corelation of CDX2 with Baseline Parameters

The association of CDX2 expression with baseline clinical and histopathological parameters is described in ►**Table 3**. Lack of CDX2 expression was significantly associated with more advanced stage of disease ($p \leq 0.0001$), rectal cancer ($p = 0.04$), higher grade of differentiation ($p = 0.045$), and presence of LVSI ($p = 0.028$). Patients with CDX2 positivity

Table 2 CDX2 expression in pathological tissue blocks

CDX2 Expression		Stage II ($n=33$)	Stage III ($n=30$)	Stage IV ($n=47$)	Total, $n=110$
CDX2	Positive ($\geq$ 5% of tumor cells +ve)	31	23	19	73 (66.4%)
	Negative ($<$ 5% of tumor cells +ve)[a]	2	7	28	37 (33.4%)
Types of scoring for CDX2 positive patients					
		Stage II	Stage III	Stage IV	$n=73$
CDX2 scoring: diffuse/focal	Diffuse	25	14	6	45 (61.6%)
	Focal	6	9	13	28 (38.4%)
CDX2 scoring in percentage	5–25%	3	4	8	15 (20.5%)
	26–50%	3	6	6	15 (20.5%)
	51–75%	15	6	1	22 (30.2%)
	$>$ 75%	10	7	4	21 (28.8%)

Abbreviations: CDX2, caudal-type homeobox 2; IQR, interquartile range.
Note: Median CDX2 positivity: 26.5%, IQR (1–70).
[a]In 37 patients who had negative CDX2 expression ($<$ 5% of tumor cells positive), 22 patients had a score of 0, i.e., no CDX2 expression at all and 15 patients had CDX2 score of 1–4%.

had relatively higher ORR as compared with CDX2 negative patients (57.1% vs. 36.8%, $p=0.22$) though statistically nonsignificant.

Treatment Particulars

Of 110 patients in the study, intent of treatment at baseline presentation was curative for 73 (66.4%) versus palliative for 37 (33.6%) patients. Definite surgery was done for 68.9% patients, 7.8% had palliative surgery, and 23.3% had no surgical intervention.

Ninety-seven (88.8%) received chemotherapy in the form neoadjuvant chemotherapy/chemoradiotherapy ($n=17$, 17.5%), adjuvant chemotherapy ($n=50$, 51.6%), or palliative chemotherapy ($n=30$, 30.9%). The chemo regimens used were: combination of capecitabine plus oxaliplatin (CapeOX) in 67 (69.1%), combination of 5FU, leucovorin, and oxaliplatin (FOLFOX) in 13 (13.4%), single agent capecitabine in 16 (16.5%), and combination of 5FU, irinotecan, and leucovorin (FOLFIRI) in 1 (1%) patients. Thirty-six (37%) patients required dose modification and there was a delay of chemotherapy between the cycles in 40 (41.2%) patients for various reasons. Among 97 patients, 50 who received neoadjuvant chemotherapy/chemoradiotherapy or palliative chemotherapy were eligible for response assessment. Out of 50 eligible patients, responses were available for 41 (82%) patients. ORR was 19 (38%), 7 (14%) had stable disease and 15 (30%) had progressive disease while in 9 (18%) response was not available, as patients were lost to follow-up. Most common adverse effects with chemotherapy were hand-foot syndrome ($n=63$, 61.2%), gastrointestinal (nausea; $n=64$, 62.1%), and hematological (anemia; $n=45$, 43.7%).

Survival Outcomes

The median follow-up of the study was 16 months. For the entire study cohort median PFS for stage II and III was not reached while for stage IV it was 15.5 months (95% confidence interval [CI] 10.4–20.5 months). Median OS for stage II and III were also not reached while for stage IV it was18.7 months (95% CI 15.3–22.1 months). For stage II, III, and IV, PFS at 2 years were 84, 68, and 25% and OS at 2 years 91, 73, and 31%, respectively, as shown in ►**Fig. 2**. The median PFS and OS were not reached for CDX2 positive tumors compared with CDX2 negative patients who had median PFS of 15.9 months (95% CI 10.3–21.4 months) and median OS of 25.8 months (95% CI 12.1–39.4 months). At 2 years, PFS and OS were 66 and 77% for CDX2 positive and 30 and 46% for CDX2 negative patients, respectively, as shown in ►**Fig. 2**.

Factors Affecting Survival Outcome

Factors associated with significantly poor PFS in univariate analysis, described in ►**Table 4**, were advanced stage (stage IV) (hazard ratio [HR] = 6.27, 95% CI: 2.14–18.30), lack of CDX2 expression (HR 2.47, 95% CI: 1.22–5.03), and higher grade of tumor differentiation (HR for grade II and III were 2.08 and 10.24, 95% CI: 0.91–4.74 and 3.46–30.30, respectively). Factors associated with poorer OS were stage IV disease (HR = 8.63, CI: 2.01–37.06), lack of CDX2 expression (HR = 2.43, 95% CI: 1.15–5.12), and higher grade of differentiation (HR for grade II and III were 2.65 and 6.04, 95% CI: 1.06–6.60 and 1.78–20.49, respectively). However, in multivariate analysis only stage and grade were independently associated with both PFS and OS.

Discussion

Risk stratification based upon prognostic markers is applied for treatment and predicting outcomes of CRCs. In early-stage colon cancer, higher grade, presence of obstruction or perforation, higher stage, and MMR deficiency are considered to harbor a poorer prognosis. Recently, CDX2, an intestine-specific nuclear transcription factor, has emerged as a new prognostic biomarker in CRC.[13,16,17] In this study, we identified the pattern of CDX2 expression in all the stages of CRC and have evaluated the association of CDX2 expression

Table 3 Correlation of CDX2 with baseline parameters and response rate

Features $n = 110$	Positive CDX2 expression ($n = 73$)	Lack of CDX2 expression ($n = 37$)	p
Age			
(a) $\leq$ 30 y ($n = 10$)	6 (8.2%)	4 (10.8%)	0.585
(b) 31–60 y ($n = 67$)	47 (64.4%)	20 (54.1%)	
(c) $>$ 60 y ($n = 33$)	20 (27.4%)	13 (35.1%)	
Gender ($n = 110$)			
(a) Male ($n = 62$)	37 (50.7%)	25 (67.6%)	0.107
(b) Female ($n = 48$)	36 (49.3%)	12 (32.4%)	
Stage of disease ($n = 110$)			
(a) Stage II ($n = 33$)	31 (42.5%)	2 (5.4%)	< 0.0001
(b) Stage III ($n = 30$)	23 (31.5%)	7 (18.9%)	
(c) Stage IV ($n = 47$)	19 (26%)	28 (75.7%)	
Site of disease: colon/rectum ($n = 110$)			
(a) Colon ($n = 77$)	56 (76.7%)	21 (56.8%)	0.047
(b) Rectum ($n = 33$)	17 (23.3%)	16 (43.2%)	
Sidedness of primary disease			
(a) Right colon ($n = 38$)	27 (37%)	11 (29.7%)	0.096
(b) Left colon ($n = 39$)	29 (39.7%)	10 (27.0%)	
(c) Rectum ($n = 33$)	17 (23.3%)	16 (43.2%)	
Differentiation ($n = 110$)			
(a) Grade I ($n = 62$)	47 (64.4%)	15 (40.5%)	0.045
(b) Grade II ($n = 34$)	18 (24.7%)	16 (43.2%)	
(c) Grade III ($n = 5$)	4 (5.5%)	1 (2.7%)	
(d) Mucinous ($n = 9$)	4 (5.5%)	5 (13.5%)	
LVSI ($n = 72$): $n = 15$, $n = 57$			0.028
(a) Absent ($n = 48$)	42 (73.7%)	6 (40%)	
(b) Present ($n = 24$)	15 (26.3%)	9 (60%)	
Response to chemotherapy (NACT/NACTRT or palliative) ($n = 40$): $n = 21$, $n = 19$			
Overall response (complete + partial responses)	12 (57.1%)	7 (36.8%)	0.22
Stable disease + progressive disease	9 (42.8%)	12 (63.2%)	

Abbreviations: CDX2, caudal-type homeobox 2; LVSI, lymphovascular invasion; NACT, neoadjuvant chemotherapy; NACTRT, neoadjuvant chemoradiotherapy.

with baseline clinical and histopathological features, response to chemotherapy, and survival outcomes. Our study has shown that lack of CDX2 expression is associated with advanced stage, higher grade, presence of LVSI, and poorer PFS and OS.

CDX2, a nuclear protein expressed by the intestinal epithelium cells throughout the gut, is essential for the proliferation and growth of epithelial cells and is frequently downregulated during tumorigenesis.[18–20] Malignant progression of colorectal adenocarcinomas is characterized by the expression of oncoprotein β-catenin[21,22] during tumor cell invasion and metastasis. CDX2 reduces the cellular proliferation and inhibits the β-catenin/T cell transcription factor transcriptional activity in colon cancer.[23,24] Lack of CDX2 is associated with loss of differentiation of gastrointestinal epithelium leading to unregulated proliferation.[25–28] Though the precise mechanisms for loss of CDX2 expression are not well characterized, low expression by IHC/gene expression has been associated with poorer prognosis, especially in early-stage CRC.[13] Since IHC is fast, reliable, and economical, we evaluated the pattern of expression of CDX2 by IHC in all stages of CRC and its association with prognosis.

There is variation in loss of CDX2 expression reported in different studies ranging from 4 to 30%, depending on the patient population, proportion of early and advanced stage colon cancers, proportion of rectal cancers, method for studying CDX2 (gene expression profile or IHC), tissue heterogeneity depending on use of tissue microarray or

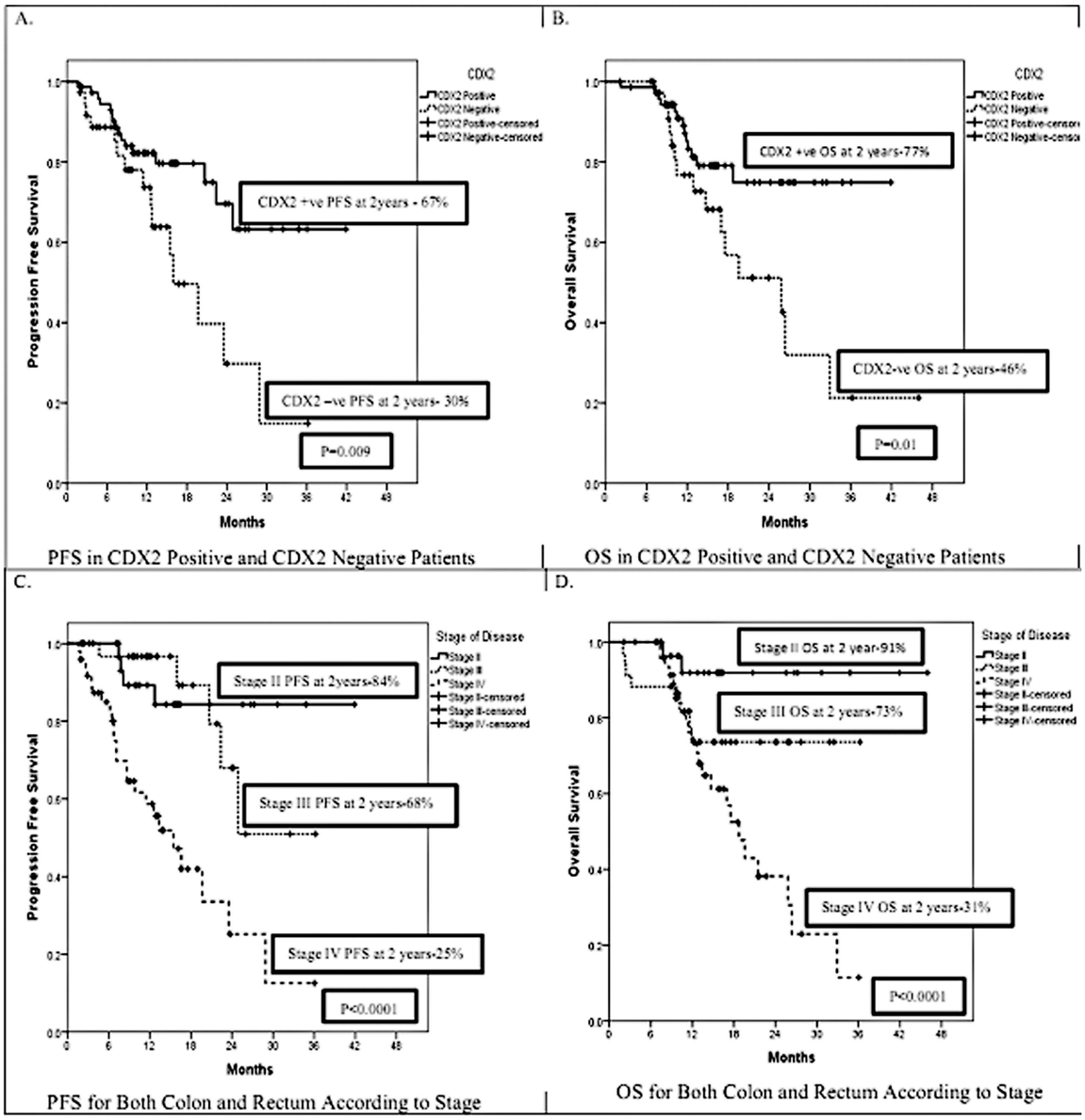

Fig. 2 (**A**) Progression-free survival for caudal-type homeobox 2 (CDX2) positive and negative patients. (**B**) Overall survival for CDX2 positive and negative patients. (**C**) Progression-free survival for stage II, III, and IV colorectal cancer. (**D**) Overall survival for stage II, III and IV colorectal cancer.

whole tissue section for IHC, and cutoff chosen for CDX2 positivity.[13,16,29] In our study, we have found a higher rate of loss of CDX2 expression (33.6%) overall, possibly because of inclusion of patients with rectal cancers, a significant proportion of patients with stage IV disease, and use of whole tissue section for IHC. Our results are comparable to the study by Baba et al who reported loss of CDX2 in 29% of patient population of all stages of CRC.[16] Notably, CDX2 expression varies with the stage of CRC showing significantly low or loss of expression in advanced stage of the disease.[16] In our study around 2% of stage II CRC lacked CDX2 as compared with 6 and 25% of stage III and IV cases. In contrast to the study by Bae et al where female gender was associated with loss of CDX2 expression,[7] we did not find any significant association with age or gender, possibly due to population heterogeneity. Disease-related parameters like higher stage of disease, rectal site, higher grade of differentiation, and presence of LVSI were significantly associated with lack of CDX2 expression in our as well as in other studies.[7,13,16,30–32] In our study, in patients receiving neoadjuvant or palliative chemotherapy, loss of CDX2 expression was not associated with response rate possibly due to small assessable number ($n = 40$) in this subgroup. Association of CDX2 expression with response rate to chemotherapy in the neoadjuvant or palliative setting has not been evaluated in any other study.

As CDX2 has an important role in impeding proliferation and promoting cellular differentiation,[33] loss of CDX2 expression is associated with poor outcomes. CDX2 loss contributes to aggressive disease behavior,[34] thereby increasing the likelihood of advanced stage.[35] In our study,

Table 4 Univairate analysis for progression-free survival and overall survival of study cohort

Features	Progression-free survival		Overall survival	
	HR (95% CI)	*p*	HR (95% CI)	*p*
Age ($n = 110$)				
(a) < 30 y ($n = 10$)	1		1	
(b) 31–60 y ($n = 67$)	0.54 (0.18–1.61)	0.27	0.49 (0.17–1.49)	0.21
(c) > 60 y ($n = 33$)	0.64 (0.19–2.10)	0.47	0.65 (0.20–2.13)	0.48
Gender ($n = 110$)				
(a) Male ($n = 62$)	1		1	
(b) Female ($n = 48$)	1.02 (0.50–2.05)	0.93	1.58 (0.75–3.33)	0.22
Stage of disease ($n = 110$)				
(a) Stage II ($n = 33$)	1		1	
(b) Stage III ($n = 30$)	1.36 (0.36–5.11)	0.64	2.68 (0.51–13.93)	0.23
(c) Stage IV ($n = 47$)	6.27 (2.14–18.30)	0.001	8.63 (2.01–37.06)	0.004
Site of disease ($n = 110$)				
(a) Colon ($n = 77$)	1		1	
(b) Rectum ($n = 33$)	1.26 (0.60–2.64)	0.54	1.13 (0.52–2.46)	0.74
Serum CEA levels ($n = 78$)				
(a) Normal ($n = 22$)	1		1	
(b) Elevated ($n = 56$)	1.89 (0.63–5.61)	0.25	3.85 (0.89–16.67)	0.07
CDX2 ($n = 110$)				
(a) Positive ($n = 73$)	1		1	
(b) Negative ($n = 37$)	2.47 (1.22–5.03)	0.01	2.43 (1.15–5.12)	0.01
Differentiation ($n = 110$)				
(a) Grade I ($n = 62$)	1		1	
(b) Grade II ($n = 34$)	2.08 (0.91–4.74)	0.07	2.65 (1.06–6.60)	0.03
(c) Grade III ($n = 5$)	10.24 (3.46–30.30)	< 0.0001	6.04 (1.78–20.49)	0.004
(d) Mucinous ($n = 9$)	3.31 (0.69–9.24)	0.16	4.92 (1.56–15.51)	0.006
LVSI ($n = 72$)				
(a) Absent ($n = 48$)	1		1	
(b) Present ($n = 24$)	1.23 (0.37–4.12)	0.72	2.14 (0.61–7.43)	0.22

Abbreviations: CDX2, caudal-type homeobox 2; CEA, carcinoembryonic antigen; CI, confidence interval; HR, hazard ratio; LVSI, lymphovascular invasion.

loss of CDX2 in CRC patients was associated with both poor PFS and OS in univariate analysis, but no significance was seen in multivariate analysis possibly due to small sample size. Independent predictors of poor survival in multivariate analysis in our patient cohort were the widely established factors of advanced stage and higher grade. Independent prognostic effect of loss of CDX2 expression on survival outcomes has diverse results across studies. Results similar to our study of the significance of loss of CDX2 with poor survival outcomes in univariate but not in multivariate analysis in CRC was observed by Baba et al and Lugli et al,[16,36] though other studies have shown that loss of CDX2 expression was independently associated with poor prognosis mostly in early-stage CRC.[7,13,37]

The limitations of our study were inclusion of a heterogeneous patient population, small sample size due to unavailability of many tissue blocks, some missing baseline information in the retrospective cohort, and short follow-up. Also, absence of MMR status for majority of cases was another main limitation. Use of whole tissue sections instead of tissue microarray, though labor-intensive, avoids tumor heterogeneity and false negative rates and this was one of the strengths of our study.

Conclusion

In conclusion, we found that loss of CDX2 expression is associated with markers of poor prognosis such as advanced disease stage, higher tumor grade, and presence of LVSI in CRC. Loss of CDX2 could possibly drive the evolution to poor biologic characteristics in the tumor. Loss of CDX2 was associated with worse PFS and OS in univariate analysis

and our results attests its role as a poor prognostic factor in CRC. However, independent prognostic role of CDX2 in survival outcomes of CRC needs to be confirmed in larger multicenter data set with relatively longer follow-up.

Authors' Contributions

Study concept and design: S.K., J.S., B.D., N.G.R. Provision of patient and study material: J.S., N.G.R., B.D., S.K., P.G., N. K.M., I.C. Performance and interpretation of IHC (immunohistochemistry): J.S., N.G.R., I.C. Data collection and analysis: J.S., N.G.R., S.K., B.D., K.K.M., I.C. Manuscript writing: J.S., S.K., B.D., P.G., N.G.R., N.K.M. Final approval of manuscript: all authors.

Note

The article has been read and approved by all authors. The requirements for authorship have been met, and all authors believe that the article represents honest work. Justification for more than six authors: This study is an interdepartmental collaboration, initiated by the Department of Medical Oncology in conjunction with the Departments of Pathology and Surgery. Authorship has been assigned to all researchers who made substantial contributions to the work, in accordance with the ICMJE authorship criteria.

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Conflict of Interest

None declared.

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Real-World Experiences of Next-Generation Sequencing in Oncology: From an Indian Multicenter Registry and Collaborative Centers

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Abstract

Background The integration of next-generation sequencing (NGS) in guiding personalized therapy for oncology faces the challenges, primarily, of cost and drug accessibility. Limited data from Indian academic centers accentuate the need for comprehensive insights into the real-world applications of NGS in oncology.

Methods The Network of Oncology Clinical Trials in India (NOCI), accessible at www.noci-india.com, compiled data on patients who underwent NGS for solid organ cancers from January 1, 2018, to December 31, 2021. This study aimed to elucidate the testing indications, sample types analyzed, and the resultant impact on patient care.

Results Analysis of data from six centers included 278 subjects, with 24 specimens (9%) excluded due to quality test failure. Tissue constituted 59.7% of specimens, blood 38.5%, and both 1.8%. Predominantly, NGS was employed for identifying *BRCA*1/2 mutations (56%) and for targeted therapy in non-small-cell lung cancer (NSCLC; 28%). Only 41 (16%) patients with other cancers underwent multigene NGS panels in pursuit of targetable mutations. Among them, 13 exhibited targetable mutations, and 3 received treatment based on NGS findings.

Conclusion This study underscores that the majority of NGS applications focused on screening for *BRCA*1/2 mutations and identifying targetable mutations in NSCLC. However, among those undergoing NGS for advanced cancers, only a limited number received personalized therapy. The findings underscore the challenges of utilizing NGS in off-label indications within resource-constrained settings.

Keywords
- hereditary cancer syndromes
- next-generation sequencing
- oncology
- targeted therapy

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Introduction

The Human Genome Project in 2003 gave us a better understanding of the molecular basis of diseases, including cancer. Further, the development of molecular oncology has ushered in the era of precision oncology.[1] The success of trastuzumab (approved in 1998 for metastatic breast cancer) and imatinib (approved in 2001 for chronic myeloid leukemia) showed that a more effective and less toxic way of treating cancer was possible, paving the way to personalized medicine (PM) and targeted therapy.[2] The Sangers method[3] and Maxwell–Gilbert method were the initial methods used to sequence genes. However, they were time-consuming, labor intensive, and financially demanding. Currently, multiple parallel testing using (NGS) has reduced the time and cost of doing these tests. NGS is being used in oncology practice for various purposes. In oncology, NGS helps detect somatic driver mutations, resistance mechanisms, quantification of mutational burden, and evaluation of hereditary cancers.[4,5] Since the last decade, use of NGS has become standard practice in the evaluation and management of several cancers. However, its use is limited in India, primarily due to cost, but also due to limited availability and access to the newer targeted agents.[6,7] The European Society for Medical Oncology (ESMO) guidelines 2020 recommend using NGS on tumor samples in advanced nonsquamous NSCLC, prostate cancers, ovarian cancers, and cholangiocarcinoma.[8] However, the practical uses of NGS may be limited in real-world settings and data from low- and middle-income countries are limited.[9,10]

Materials and Methodology

Study Design

We conducted a retrospective, multicenter descriptive study under the aegis of the Network of Oncology Clinical Trials India (NOCI) and other collaborative centers. NOCI is a cooperative research network developed with a grant from the Department of Biotechnology, Government of India, having six member institutes from all parts of the country. Electronic and paper databases from these institutes were checked from January 1, 2018 to December 31, 2021, and data on the NGS test done were collected. Anonymized data were obtained after ethical approval from each institute and the data were collected in a spreadsheet. Details on patient demographics, biopsy type, prior lines of systemic therapy, type and number of mutations, NGS platform used, genome-directed therapy received, and rationale behind the rejection of sequencing-directed therapies were extracted from data capture forms. The proportion of profiled patients receiving sequencing-directed therapies and the reasons for declining targeted therapies were then evaluated using descriptive statistics.

Inclusion and Sample Size

For this study, we included all adult patients (>18 years) with nonhematological cancers who had undergone NGS testing for any indication and for whom a verifiable report was available. As it was an observational retrospective descriptive study, it was planned to include all patients from electronic and paper databases for whom NGS reports were available from January 1, 2018 to December 31, 2021, and no separate sample size calculation was applicable.

Primary and Secondary Outcome

To assess the utilization and impact of NGS in the management of solid organ cancers in resource-constrained settings in India, specifically focusing on indications for testing, types of samples tested, and the presence of druggable mutations.

Statistical Analysis

Data were anonymized and recorded in MS Excel spreadsheet program (2019) and used for analysis. Before interpreting the results, the data were cleaned, checked for accuracy, missing values noted, labeled appropriately, sorted, and recoded as required.

Descriptive statistics were elaborated in the form of means/standard deviations and medians/interquartile ranges (IQRs) for continuous variables, and frequencies and percentages for categorical variables.

Ethics

The study was done after ethics committee approval, vide Ref: BMHR-IECCMCL/0322-115/Apprvl/NEXT-G/Med-Onco, dated March 19, 2022. Waiver of consent was taken as it was a noninterventional retrospective descriptive study and patients' details were anonymized. The study was done in accordance with the ethical standards of the ICH GCP ICMR guidelines. The study was registered in Clinical Trials Registry-India (CTRI), vide number: CTRI/2022/01/039233

Results

A total of 278 patients had undergone NGS analysis during the study period. The median age of the patients was 55 years (range: 27–82 years) and females ($N = 183$) accounted for 65.3% of the patients (►**Table 1**). Specimens used for testing were tissue ($N = 166$; 59.7%), blood ($N = 107$; 38.5%), and a combination of blood with tissue ($N = 5$; 1.8%). Of the 278 samples sent for testing, 9% ($N = 24$) were rejected as they failed the quality testing and only 254 samples were analyzed (►**Fig. 1**). The majority of the patients had advanced stage ($N = 185$, 66.5%) and test was ordered upfront at diagnosis in 178 patients (64%). One hundred and ninety-nine patients (78.6%) had tested for limited gene panels of less than 50 genes. The most common indications for testing were lung cancer ($N = 72$, 28.3%), ovarian cancer ($N = 65$, 25.6%), and breast cancer ($N = 54$, 21.3%). For patients with lung cancer ($N = 72$), the use of NGS identified 56.9% ($N = 41$) specimens with targetable mutations leading to the use of targeted therapy in 36.1% ($N = 26$) patients (►**Table 2**). Among the patients in which NGS was tested for off-label indications ($N = 41$, 16.1%), 13 patients were found to have targetable mutations and 3 patients were receiving targeted therapy based on the NGS reports (►**Table 3**).

Table 1 Baseline characteristics

Variable	Number	Frequency (%)
Gender ($N = 278$)		
Male	95	34
Female	183	66
Type of tissue used ($N = 278$)		
Tissue	166	59.7
Blood	107	38.5
Blood and tissue	5	1.8
Stage ($N = 278$)		
1	8	2.9
2	19	6.8
3	66	23.8
4	185	66.5
Clinical scenarios of NGS testing ($N = 278$)		
Upfront at diagnosis	178	64
Post 1st line	41	14.7
Post 2nd line	59	21.3
No. of genes tested ($N = 254$)		
<50 gene assays	199	78.6
50–150 gene assays	16	6.32
150–350 gene assays	33	13
>350 gene assays	6	2
Types of cancer ($N = 254$)		
Lung cancer	72	28.3
Ovarian cancer	65	25.6
Breast cancer	54	21.3
Pancreaticobiliary cancers	22	8.6
Prostate cancer	16	6.3
Gastrointestinal cancer	5	2.0
Gynecological cancer excluding ovary	4	1.6
Genitourinary malignancies	4	1.6
Others[a]	12	4.7

[a]Other cancers included dual primary ($N = 2$), carcinoma unknown primary ($N = 2$), head and neck squamous cell carcinoma ($N = 2$), sarcomas ($N = 3$), skin carcinoma ($N = 1$), and thyroid cancer ($N = 2$).

However, with regard to testing for hereditary cancer syndromes, the most common indications were for breast and ovarian cancers ($N = 119$, 84.3%), followed by prostate and pancreatic cancers ($N = 22$, 15.6%). Out of the 141 blood and tissue samples analyzed for hereditary cancer syndromes, 25% ($N = 35$) samples were positive for *BRCA1* and *BRCA2*, which led to the use of poly-ADP ribose polymerase (PARP) inhibitors in 43% ($N = 15$) patients (►**Table 4**). The 106 samples considered negative included 5 samples with variants of unknown significance (VUS).

Discussions

The results of this study provide valuable insights into the real-world experiences of the use of NGS in the field of oncology from government and academic centers that deal with patients having resource constraints. Compared to other studies where about 8 to 20% samples failed the NGS test,[9,11] our study also showed that in 8.6% samples the test could not be done as the tissue did not have sufficient deoxyribonucleic acid (DNA) to run the test. This is an important factor in ordering these tests as the quality and quantity of tissue play a role in obtaining optimal results. In our observation, the most common indication for ordering NGS-based assay is for hereditary cancer syndromes and for lung cancer.[6,9] This is in accordance with the general practice and worldwide data, as they have high chance of being positive and also have easily accessible targeted agents. Moreover, identification of hereditary *BRCA* mutations has significant implications for the patient and the family. A point to note while testing NGS in hereditary cancer syndromes is that in case the physician is using limited gene test for BRCA1 and BRCA2 only, about 5% patients may be missed if they have large deletions and duplications, which can be tested by multiplex ligand-dependent probe amplification (MLPA). Also, it is important to offer pre- and posttest counseling as these tests have varied implications with regard to treatment, outcomes, cascade testing, psychological issues, and confidentiality. Unfortunately, there are no Indian guidelines for these; also there are limited quality control practices.

The use of limited gene panels is common in India as the testing is restricted to genes that are druggable. This could also account for the fact that the majority of the patients in our study underwent limited gene panel in lung cancer and for hereditary cancer syndromes. Our series showed prevalence of 25% for *BRCA1* and *BRCA2* genes using limited panel testing; it needs to be seen if the use of multigene panels covering for other genes involved in hereditary cancer syndromes would have made any significant impact to our population of patients. For those identified with *BRCA*1 and BRCA2 mutation, 43% ($N = 15$) received PARP inhibitors during their course of treatment. This number is bound to increase as the indications for using PARP inhibitors are increasing and the cost of PARP inhibitors are coming down with more generics being available. With regard to the use of NGS for lung cancer, our study shows the feasibility of using NGS platforms as it may be convenient to test multiple targets with limited tissue available. Most of the patients in our study had used limited gene testing for lung cancer, and it is common practice in the NOCI centers to use alternative methods for testing targetable mutations in lung cancer as it is cheaper. With regard to non-BRCA and non-lung-cancer subjects, our series shows limited number of patients. This could be due to the limited options for treating these patients outside of clinical trials in resource-constraint settings like ours. The NOCI network comprises six centers, of which two are central government institutes, two are charitable minority institutes, and two private academic

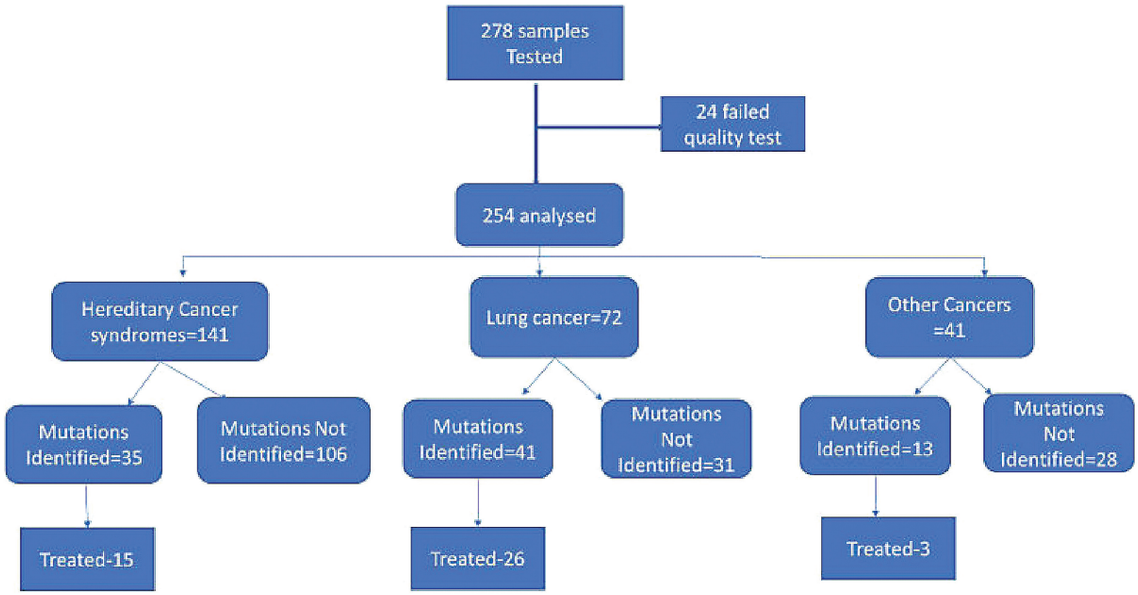

Fig. 1 Consort diagram.

Table 2 Targetable Mutations identified in lung cancer ($N = 72$)

Diagnosis	Mutation	Incidence	Percentage
Adenocarcinoma	*EGFR*	21	29
Adenocarcinoma	*ALK*	7	9.72
Adenocarcinoma	*KRAS*	4	5.5
Adenocarcinoma	*BRAF*	3	4.1
Adenocarcinoma/ squamous[a]	*RET*	3	4.1
Adenocarcinoma	*HER2*	2	2.8
Adenocarcinoma	*MET*	1	1.4

Abbreviations: *ALK*, anaplastic lymphoma kinase translocation; *EGFR*, epidermal growth factor receptor; *HER2*, human epidermal growth factor receptor 2; *KRAS*, Kirsten rat sarcoma; *MET*, mesenchymal epithelial transition factor receptor; *RET*, rearranged during transfection.

[a]*RET* was identified in one case of squamous cell carcinoma. 72 cases were analyzed, of which 66 were adenocarcinomas, 3 undifferentiated carcinomas, 2 squamous cell carcinomas, and 1 epithelioid hemangioendothelioma.

institutes. None of these centers had in-house facilities for performing NGS at the time of the study and samples had to be outsourced. Only one academic private institute had government funding for testing *BRCA1* and *BRCA2* mutations and this could have been the reason for having more patients testing for *BRCA1* and *BRCA 2*. With regard to off-label use of targeted therapy using NGS, durable clinical benefit was seen in only one patient. This patient had *Her2/Neu* driver mutation in a metastatic gallbladder carcinoma (►**Table 3**). A similar study from India showed the limitations of performing NGS testing in off-label indications.[6] That study had a different cohort of subjects and had more representation from the corporate settings. Our cohort dealt with patients presenting in resource-constraint settings and highlights the limitations of asking for these tests in the first place. Even in prospective studies using NGS for patients in advanced cancer, the benefit of using NGS for off-label indication ranges from 5 to 7%.[12–14] Some studies show that when NGS was done for identifying treatment options, most

Table 3 Off-label targets identified by NGS and patterns of treatment

Cancer type	Mutations identified	Targeted agents used as per NGS report (yes/no)	If yes, name of agent used	Summary
Carcinoma gall bladder	*TP53*, *ATR*, *JAK2*, *AIRD1A*, *ERBB4*	No		12# FOLFOX-metabolic CR for 2 y and disease relapsed and passed away before treatment
Carcinoma gall bladder	*HER2*	Yes	Trastuzumab	Post 6# gemcitabine + cisplatin 1st line PFS 6 mo, 2nd line FOLFOX + trastuzumab. PFS 1.2 y in the 2nd line with chemotherapy, progression

(*Continued*)

Table 3 (*Continued*)

Cancer type	Mutations identified	Targeted agents used as per NGS report (yes/no)	If yes, name of agent used	Summary
Carcinoma stomach	*BRAF V600E and TMB > 40 TMB >20 mutation/Mb*	Yes	Trametinib + vemurafenib, followed by pembrolizumab	Post 6# FOLFOX, progression, post 2nd line docetaxel 2# progression, then 2 mo trametinib + vemurafenib progression and then 2# pembrolizumab and passed away. No response to both agents in 2nd and 3rd lines
Bladder carcinoma	*TMB > 33 mutation/Mb*	Yes	Atezolizumab	No response in the first-line single agent 3# and succumbed to disease
Mediastinal germ cell tumor	*TMB >20 mutation/Mb, KRAS, TP53, ATR, ARID1A*	No	–	NA[a]
Carcinoma of unknown primary	*BRAF*	No	–	NA[a]
Pancreas	*TMB >20 mutation/Mb, KRAS*	No	–	NA[a]
Carcinoma stomach	*MET* amplification	No	–	NA[a]
Suspected GIST/ carcinoma unknown primary	*CKIT*	Yes	Imatinib	On imatinib for 2 y with partial response
Carcinoma endometrium	*FGFR2*	No	–	NA[a]
Carcinoma of unknown primary	*TMB > 15 mutation/Mb*	No	–	NA[a]
Cholangiocarcinoma	*TMB*-30 *mutation/Mb* and *KRAS*	No	–	NA[a]
Gingivobuccal sulcus	*TP53 and ERBB2*	No	–	NA[a]

Abbreviations: *ATR*, ataxia telangiectasia and Rad3; *AIRD1A*, AT-rich interactive domain-containing protein 1A; *ERBB4*, receptor tyrosine-protein kinase erbB-4; *FGFR2*, fibroblast growth factor receptor 2; GIST, gastrointestinal stromal tumor; *HER2*, human epidermal growth factor receptor 2; *JAK2*, Janus kinase 2; *KRAS*, *Kirsten rat sarcoma*; *MET*, mesenchymal epithelial transition; NGS, next-generation sequencing; *TP53*, tumor protein 53; *TMB*, tumor mutational burden.
[a]Not applicable -these subjects did not receive treatment based on NGS and received standard of care.
[#]cycles of chemotherapy.

Table 4 Patterns of hereditary syndromes testing ($N = 141$)

Type of cancer	Incidence	Frequency
Ovary and breast	119	84.3%
Prostate cancer	11	7.8%
Pancreaticobiliary	11	7.8%

patients could be enrolled in clinical trials.[15] That is an important point to note as we may have limited options in our setting and it is important to develop good research facilities doing ethical work. Our study has major limitations as it was a retrospective study and had a heterogenous population of patients undergoing various types of NGS testing. Also, an important point to note is the role of developing molecular tumor boards and see if the inputs would lead to better outcomes and results. It is important for the oncologist to understand and select the right patients based on the clinical profile and general condition and select the right panel when planning these tests in resource-constraint settings.

Conclusion

Our study offers practical insights into the real-world impact of using NGS in oncology in resource-constraint settings. The predominant use of NGS in resource-constrained Indian hospitals is mainly in lung and ovarian cancers where some access to targeted agents may be possible with the use of generics and support programs.

Patient Consent

Waiver of consent was taken as it was a noninterventional retrospective descriptive study and patients' details were anonymized.

Conflict of Interest
None declared.

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Histopathological Spectrum of Metaplastic Carcinoma of the Breast: A Rare Case Series and a Review of Literature

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Abstract

Introduction Metaplastic breast carcinoma (MBC) is one of the rare varieties of breast cancers (BCs) accounting for 0.2 to 2% of diagnosed cases. The tumor is known for its aggressive behavior with a large size at the time of diagnosis and rapid propagation.

Objectives The study aimed to evaluate all cases of MBC diagnosed over 4 years at a tertiary care institute and classify them according to the WHO classification of breast tumor (5th edition).

Materials and Methods All cases of MBC diagnosed in the last 4 years were reviewed retrospectively. Slides were prepared for both histopathological and immunohistochemical analyses. Relevant data were recorded.

Results All seven patients included in the study were females with aged between 39 and 61 years. The mean size of the tumor mass was 7.14 ± 1.41 cm. None of the cases showed nodal involvement. The most common histological subtype was squamous cell carcinoma (3, 42.8%), two cases were MBC with heterologous differentiation (28.5%), and one case each of adenosquamous carcinoma and spindle cell carcinoma (14.2%) was diagnosed. All the cases were p63 positive and ER, PR, HER2/neu, CD34, and CD10 negative. Additional immunohistochemical markers were used to rule out the relevant differentials, whenever required.

Conclusion This study aims to provide an account of the cases of MBC encountered in the last 4 years in the institute. This would be helpful in future diagnosis and treatment of this rare and prognostically poor subtype of BC.

Keywords
- metaplastic breast carcinoma
- histopathology
- immunohistochemistry.

Name of the department and institution to which the work is attributed: *Department of Pathology, Medical College, Kolkata, West Bengal, India.*

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Introduction

Breast cancer (BC) is one of the most common types of malignancy among the female population all over the world as well as in our country. However, metaplastic breast carcinoma (MBC) is one of the rare varieties encountered in day-to-day practice.[1] The rarity of this tumor has kept this entity unknown to us for a long time until it was first described and discovered in 1973.[2] The metaplastic carcinoma accounts for 0.2 to 1% of all BCs.[3,4] It was in the year 2000 that the World Health Organization (WHO) recognized MBC as a distinguished entity.[5]

In the most recent edition of WHO classification of breast tumors, MBC has been defined to be a "heterogeneous group of invasive breast tumors characterized by differentiation of neoplastic epithelium towards squamous cells and/or mesenchymal looking elements including but not restricted to spindle, chondroid and osseous cells."[6] The pathogenesis and molecular mechanisms are still unclear in this case and somehow considered to be different from other forms of BCs.[7] Epithelial mesenchymal transition genes might have some key role in pathogenesis.[8] The tumor is known for its aggressive behaviors having large size at the time of diagnosis and rapid propagation to a worse outcome.[9] Despite being aggressive, the tumors are often node negative and most of the cases act as basal tumors being triple negative on immunohistochemistry (IHC).[10]

In the present study, retrospective analysis of all cases diagnosed in a tertiary care center as MBC in a span of 4 years has been done, with emphasis on their histological morphologies and immunohistochemical expression to classify them as per the recent WHO classification. This classification was done to help better understand this rare entity, which would guide the diagnosis and appropriate classification of such tumors and ultimately assist in rendering better patient care.[6]

Materials and Methods

A retrospective observational study was undertaken. Nonprobability sampling technique was employed and the sample size was 7.

Records of specimens received in the department of pathology for the last 4 years were studied and specimens of the breasts were shortlisted. The total number of breast specimens was found to be 457. From those specimens, cases that were rendered the diagnosis of MBC were identified and included in the study. Seven cases were found to be diagnosed as MBC. Relevant medical records of those seven patients were retrieved from the archives. All other cases were excluded from the present study.

The following relevant data were noted for each patient: age and sex of the patients, symptoms, signs, radiological data, surgery performed and gross findings of the specimen received such as site, size, and appearance of the tumor. The paraffin blocks of the specimens that were preserved in the department of pathology were retrieved and then slides were prepared for histological and immunohistochemical staining.

Hematoxylin and eosin stained slides were used for histological evaluation as per the diagnostic criteria per WHO classification of breast tumors (5th edition).[6] All cases were stained with the following immunohistochemical markers: estrogen receptor (ER), progesterone receptor (PR), HER2/neu, Ki-67, p63, CD10, and CD34. Paraffin blocks were sectioned at 5-µm thickness and then deparaffinized and incubated with a panel of antibodies (ER—PathnSitu, clone EP1; PR—PathnSitu, clone EP2; Her2/neu—PathnSitu, clone PRM116; Ki-67—PathnSitu, clone MIB1; p63—PathnSitu, clone 4A4; CD10—PathnSitu, clone EP206; CD34—PathnSitu, clone EP88). Cases that showed positive expression of p63 and negative expression of CD34 were confirmed to be MBC.[6] Two cases that showed heterologous mesenchymal components were further stained with the following antibodies: vimentin (PathnSitu, clone EP21), S100 (PathnSitu, clone EP32), and Pan CK (PathnSitu, clone EKHP). IHC staining positivity was considered in cases of unequivocal expression of markers in $\geq$1% of tumor cells.[9]

All the findings were meticulously tabulated and analyzed.

Inclusion and exclusion criteria: Cases of MBC were retrieved from the archives. The patients were contacted and they were included in the study only after they provided written informed consent. Cases other than those of MBC were excluded from the study.

Primary and secondary outcomes: The details of MBC cases were explored and elucidated.

Statistical analysis: The statistical analysis is not relevant to the present study.

Ethics: The study was conducted following approval by the Institutional Ethics Committee (Ref. no.: MC/KOL/IEC/NONSPON/1291/03/22, dated March 16, 2022). The patients were included in the study only after obtaining their written informed consent. The study protocol has been approved by the institute's committee on human research. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Results

All the patients included in the present study were females and their ages varied between 39 and 61 years. The mean age of the patients was 50.28 $\pm$ 7.62 years. The specimens of the breast had been received following modified radical mastectomy. Each of the patients presented with painless breast mass at the time of diagnosis. Five cases had a mass in the left breast and two cases presented with a right breast mass. Most of the women (6, 85.7%) were in the postmenopausal age group (►**Table 1**)

Preoperative core biopsy findings were available in six of seven patients (85.7%) in the present study. All six cases were reported as MBC. Four of these cases (66.7%) were subtyped as squamous cell carcinoma (SCC), whereas subtyping were not been done for the other two (33.3%). One of the cases reported as SCC on core biopsy was later rendered the

Table 1 Clinicopathological characteristics of cases of metaplastic breast carcinoma

Case	Age (y)	Laterality	Gross size (cm)	Histological subtype	Immunohistochemical analysis[a]	Pathological stage
1	46	Left	6.4	MBC-SCC	p63, CK5/6 positive	pT3N0
2	61	Left	3	MBC-SCC	p63, CK5/6 positive	pT2N0
3	58	Left	7.2	MBC with heterologous differentiation	p63, pan CK, Vimentin, S100 positive	pT3N0
4	39	Right	4.5	MBC-adenosquamous carcinoma	p63, CK5/6 positive	pT2N0
5	43	Left	5.4	MBC with heterologous differentiation	p63, pan CK, vimentin, S100 positive	pT3N0
6	56	Right	20	MBC-spindle cell carcinoma	Vimentin, SMA, p63 positive; CD10, CD34 negative.	pT3N0
7	49	Left	3.5	MBC-SCC	p63, CK5/6 positive	pT2N0

Abbreviations: MBC, metaplastic breast carcinoma; SCC, squamous cell carcinoma.
[a]All cases were negative for ER, PR, Her2/neu.

diagnosis of adenosquamous carcinoma after mastectomy. None of the seven cases received neoadjuvant chemotherapy and were treated with upfront surgery (modified radical mastectomy).

Gross examination findings of the specimens revealed that the tumor sizes varied from as small as 3 cm to as large as 20 cm in maximum diameter. The mean size of the tumor mass was 7.14 ± 5.42 cm.

On microscopic examination, the tumors were diagnosed as MBC and subtyped and graded as per the WHO classification of breast tumors (5th edition).[6]

Axillary lymph node examination of all cases showed no nodal involvement. The most common subtype was SCC, which was found in three cases (42.8%) and two cases (28.5%) were diagnosed as metaplastic carcinoma with heterologous differentiation showing chondroid areas. One case each was diagnosed as adenosquamous carcinoma (14.2%) and spindle cell carcinoma (14.2%; ►**Figs. 1** and **2**). Ductal carcinoma in situ (DCIS) was noted in two of the seven cases (28.6%).

Three cases diagnosed as SCC showed atypical squamous cell proliferation within the stromal component of the breast tissue with the formation of cyst-like spaces lined by

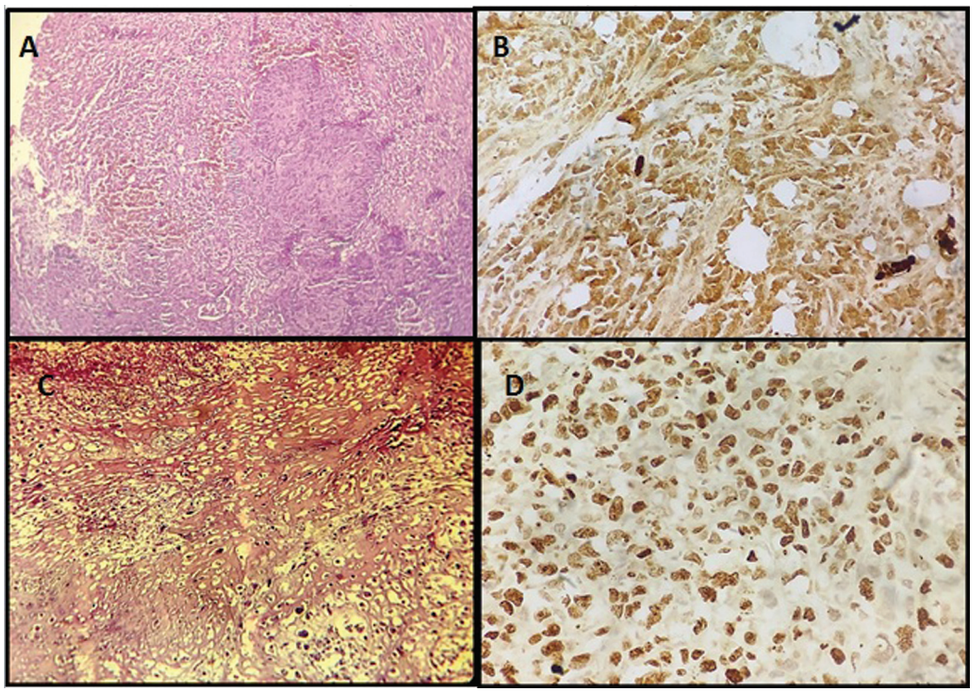

Fig. 1 (**A**) Microscopic appearance of metaplastic breast carcinoma (MBC) of squamous cell carcinoma (SCC) subtype (hematoxylin and eosin [H&E], ×100). (**B**) Positive expression of p63 immunostain in SCC subtype of MBC (×400). (**C**) Microscopic appearance of MBC with heterologous (cartilaginous) differentiation (H&E, ×400). (**D**) Ki-67 expression of 40% by the tumor cells of MBC.

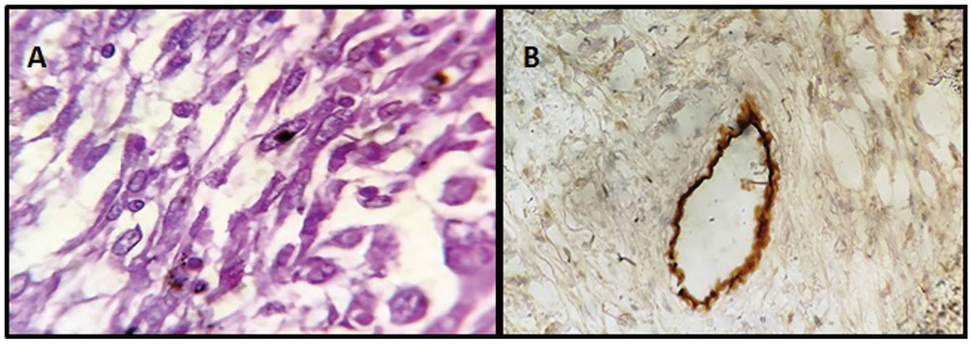

Fig. 2 (**A**) Microscopic appearance of the spindle cell carcinoma subtype of metaplastic breast carcinoma (MBC; hematoxylin and eosin [H&E], ×400). (**B**) CD34 negative cells in spindle cell carcinoma (internal control: blood vessel showing positivity for CD34; ×400).

squamous cells with hyperchromatic, pleomorphic nuclei. No evidence of primary SCC elsewhere was found, which ruled out the possibility of metastatic SCC of the breast. SCC type of metaplastic carcinoma showed the presence of p63 and CK5/6 IHC stains.

Two cases showed heterologous differentiation of its components. One of them revealed proliferation of mature cartilaginous tissue, which represented mesenchymal differentiation admixed with epithelial glandular components. The cartilaginous component of the tumor showed positive IHC staining with vimentin and S100. In another case, osseous along with rhabdoid differentiations were found. The osseous component showed positive IHC staining with S100 and the rhabdoid component was positively stained with vimentin and desmin.

Adenosquamous carcinoma was diagnosed in one case showing histological features of infiltrative small round glandular structures with focal areas of squamous differentiation with a desmoplastic stroma. Adenosquamous carcinoma type of MBC showed positive p63 and CK5/6 staining in epithelial cells of glandular structures and areas of focal squamous differentiation.

One case, however, showed proliferation of atypical pleomorphic spindle cells with hyperchromatic nuclei and prominent nucleoli arranged in fascicles and sheets with invasion of stromal tissue. The case was differentiated from primary stromal sarcoma and other spindle cell neoplasms (e.g., malignant phyllodes tumor) of the breast by using the relevant immunohistochemical markers and diagnosed as spindle cell carcinoma. Spindle cell carcinoma showed positivity with vimentin, SMA, and p63 stains, and the cells were negative for CD10 and CD34 stains.

Pathological staging of all seven cases was done. Four of the cases (57%) showed pathological stage 3 (pT3). These included two cases of MBC with heterologous differentiation, one case each of SCC and spindle cell carcinoma. Two cases (28.6%) of SCC and one case (14.4%) of adenosquamous carcinoma were of pathological stage 2 (pT2). All cases showed an N0 status.

Immunohistochemical analysis showed that all seven cases were absent for detection of ER, PR, and Her2/neu. CD34 and CD10 were also negative. All the cases showed positivity for p63. High Ki-67 expression (average score: $46 \pm 0.25\%$) was noted in all cases except one case of SCC type of MBC where the Ki-67 expression was $\leq 1\%$.

Discussion

In the present study, cases of MBC were evaluated with respect to the various clinicopathological parameters. Due to the lack of characteristic imaging patterns and histological similarities between various malignant breast lesions, MBC is difficult to diagnose. Postoperative histopathological and immunohistochemical analyses are the main modalities of diagnosis at present.

MBC is one of the rarest forms of BC. There is a dearth of reports of MBC cases from the eastern regions of the country. In the current study, the incidence rate was 1.53%, which was similar to studies done by Nelson et al and Znati et al.[4,11] It was also similar to the incidence rate mentioned by the WHO classification of breast tumor.[6] The age group of the patients of the present study varied between 39 and 61 years, which was corroborative with other studies.[12–14] A study by Pezzi et al showed MBC occurs mostly in postmenopausal women with a rapid increase in tumor size, advanced stage at the time of diagnosis, and hormonal receptor negative status.[15] These findings were comparable with those of the current study. The most common site of tumor mass according to this study was the left breast, which was similar to study done by Salimoğlu et al.[16]

The gross tumor sizes varied from as small as 3 cm to as large as 20 cm in maximum diameter. The mean size of the tumor mass was 7.14 ($\pm$ 1.41) cm in this study. These findings are similar to other studies.[11,17] A study by Gultekin et al showed the relationship between tumor size, survival rate, and recurrence.[14]

SCC (42.8%) was the most common histological type of MBC diagnosed in the present study, followed by two cases of metaplastic carcinoma with heterologous differentiation (28.5%) and one case each of adenosquamous carcinoma (14.2%) and spindle cell carcinoma (14.2%). A study by Salimoğlu et al showed similar findings, with SCC being the most common histological type of MBC.[16]

In comparison to invasive breast carcinoma, NOS (not otherwise specified) of similar size and grade, lymph node metastases are quite rare in MBC.[18] In the current case series,

axillary lymph node dissection was done in every case and all of them showed negative lymph node involvement. These findings corroborated with other studies.[14,17]

No single immunohistochemical marker is constantly positive in all cases of MBC. Hence, use of a panel of IHC markers is essential. According to Rakha et al, at least one marker of epithelial differentiation is expressed by most MBCs.[19] The majority of MBCs are p63 positive and all seven cases of the present series showed p63 positivity. This result was similar to other studies.[20,21] CD34 was consistently negative in all seven cases excluding phyllode tumors as a differential diagnosis. However, CD10 is known to be positive in 50 to 70% of MBC cases, although all the tumors turned out to be CD10 negative in the current series.[6]

IHC showed all of the tumors were ER, PR, Her2/neu, CD34, and CD10 negative. These findings were similar to the study done by Yamaguchi et al.[9]

The limitations of the present study were the paucity of cases and limited resources, which restricted the panel of IHC markers used. It was beyond the scope of this study to include sophisticated investigations like tumor genomics/comprehensive genomic profiling.

Conclusion

MBC is a rare entity of BC associated with a worse prognosis than other types of BC, although low-grade adenosquamous carcinoma shows a better prognosis than other types of MBC. The diagnostic difficulty of MBCs is due to its morphological and molecular heterogeneity and lack of a marker, which is consistently expressed in all cases. The present study aims to provide a comprehensive idea about the common histological subtypes and immunoprofile of MBC cases in routine practices.

Patient Consent

Written informed consent was obtained from the patients included in the study.

Conflict of Interest

None declared.

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Treatment-Related Toxicity Profile in Children with Acute Lymphoblastic Leukemia/Lymphoblastic Lymphoma during Induction Chemotherapy: Prospective Insights from a South Indian Cohort

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Abstract

Introduction There is limited data on treatment-related toxicity and mortality in children with acute lymphoblastic leukemia (ALL) in resource-limited settings, which is important for anticipation and better prophylactic care.

Objectives This study aims to determine the proportion of children with ALL/T- lymphoblastic lymphoma (T-LL) developing treatment-related toxicity during induction chemotherapy, to study the proportion of mortality, the cumulative duration and frequency of treatment delays, and frequency of treatment protocol modifications.

Materials and Methods It was a prospective cohort study where all consecutive children aged between 1 month and 15 years with ALL/T-LL were included. They were followed up during induction chemotherapy and toxicities were recorded, graded as per the Common Terminology Criteria for Adverse Events grading, and analyzed.

Results One-hundred and one children with median age of 6 years (interquartile range: 3, 11) were enrolled. A total of 696 toxicities were recorded among 101 children, of which 418 (60.06%) were of grades 3 and above, which were studied. Number of grades 3, 4, and 5 toxicities were 236 (33.91%), 176 (25.29%), and 5 (0.72%), respectively; of which 73.2% were hematological and 26.8% were nonhematological. Sixty-five treatment breaks were observed in 51 children (50.49%), with majority (34%) occurring in week 3. Protocol modification was observed in 10 (9.9%) due to toxicities and 9 (8.9%) required intensive care admission. Our induction mortality rate was 8.9%, of which infection-related mortality was observed in 4.9%. A statistically significant association was observed between risk group and toxicities of grade ≥ 3, with the nonstandard risk group exhibiting 3.21 times higher odds of experiencing these toxicities compared with the standard risk group.

Conclusion The results obtained from our study showed the prevalence and profile of treatment-related toxicities, with hematological toxicities being the most prevalent

Keywords

- acute lymphoblastic leukemia
- lymphoma
- induction chemotherapy
- treatment delay
- resource-limited settings

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and infections and treatment delays being major concerns. High-risk disease was associated with increased toxicity, reinforcing the importance of proactive management strategies.

Introduction

Acute lymphoblastic leukemia (ALL) was earlier considered a highly fatal disease attributed to disease progression and relapse, necessitating intensive chemotherapy. With the invention of aggressive chemotherapy, the overall survival rates increased; disease- and relapse-related mortality came down; however, toxicity-related mortality (TRM) increased. To overcome this came the concept of risk-adapted therapy, which provides treatment based on risk stratification and minimal residual disease (MRD) analysis, to lessen the burden of treatment-related complications and mortality without compromising the efficacy of therapy. While advancements in risk-adapted therapy, molecular diagnostics, and supportive care have improved survival rates to over 80% in developed countries and 70% in India,[1] treatment-related toxicity (TRT) remains a major challenge.

Incidence of TRM in developed countries is $< 3\%$, but in developing countries like India, TRM ranges between 10% and 23%, with infections being the leading cause of TRM.[2] TRM during induction ranges between 8.6% and 10.6%.[2] TRM is just a tip of the iceberg.

Challenges in treating childhood ALL in India include treatment-related complications, lack of risk-adapted protocols, infrastructure limitations, delayed diagnosis, poor chemotherapy tolerance, and malnutrition.[3] Implementing risk-stratified therapy, early diagnosis, and adequate supportive care can mitigate these risks. Recent protocols including the Indian Collaborative Childhood Leukemia (ICiCLe) protocol now provide tailored therapy utilizing disease genetics and MRD analysis to improve outcomes, while minimizing toxicity and costs.[4]

Although toxicity profiles of chemotherapy regimens in ALL are well-studied in Western population, they may not be directly applicable to developing countries due to differences in socioeconomic factors, nutritional status, and prognostic factors.[2,3] The incidence and profile of TRT, not amounting to mortality, is not studied in detail from developing countries, which is essential for early intervention and improved management. Limited data exists on TRTs in Indian children, particularly in South India. This study aims to evaluate the toxicity profile in children with ALL/T-LL receiving induction chemotherapy at a tertiary care center in South India.

Materials and Methods

Study Design and Settings

This was a prospective cohort study conducted in a tertiary care center in South India between July 2022 and June 2024.

Sample Size

Considering a reported 20% toxicity rate during induction chemotherapy in ALL,[5] a sample size of 130 was calculated for 7% absolute precision; with 5% attrition, the final estimated sample size was 136.

Primary Objective

To determine the proportion of children developing TRT during induction chemotherapy for ALL/T-LL aged 1 month to 15 years.

Secondary Objectives

In children with ALL/T-LL aged 1 month to 15 years during induction chemotherapy,

- To study the cumulative duration and frequency of treatment delays and treatment protocol modification.
- To study the proportion of mortality in the study cohort.

Inclusion Criteria

All consecutive children aged 1 month to 15 years with newly diagnosed ALL/T-LL who are admitted for induction chemotherapy.

Exclusion Criteria

- Children who expired prior to initiation of induction chemotherapy due to disease-related complications.
- Those who left against medical advice prior to or during induction chemotherapy.

Expected Outcomes

Primary Outcome

The primary outcome of the study was the proportion of children with ALL/T-LL developing grade 3 or higher TRT during induction chemotherapy.

Secondary Outcomes

The secondary outcomes included:

1. Induction mortality rate
2. Cumulative duration and frequency of treatment delays during induction chemotherapy
3. Proportion of children requiring protocol modifications during induction chemotherapy
4. Association between risk group and incidence of grade ≥ 3 toxicities

Patient demographics, clinical characteristics, and laboratory details at the time of admission for induction chemotherapy were documented using a structured proforma. The diagnosis of ALL/T-LL and risk stratification were based on

standardized protocols, incorporating peripheral blood and bone marrow examination, cerebrospinal fluid analysis, flow cytometry, cytogenetics, and molecular studies.

Key data collected included demographic details, clinical examination findings, anthropometric measurements, malignancy details, risk group, treatment protocol adapted, and drugs administered, including doses. Chemotherapy was administered according to the Berlin-Frankfurt-Munster-95 or ICiCLe protocols as decided by the medical oncology team. Investigations were performed and toxicities were managed, if any, as part of routine management.

Children were followed up at regular intervals (twice weekly) from diagnosis till the end of induction chemotherapy. TRTs were recorded, along with supportive care interventions such as transfusions and critical care support, if any. Relevant investigations, including hematological, radiological, microbiological, and biochemical tests, were systematically documented.

The treatment breaks, timing of these breaks, the reasons for the same, and treatment/protocol modifications were noted. Cumulative treatment breaks at the end of induction were collected. Response assessments at the end of induction chemotherapy were recorded by bone marrow examination and MRD analysis. All data were collected from inpatient hospital records to ensure comprehensive analysis and evaluation. In the children who expired, the cause of mortality was noted and contributing factors were studied.

The degree of organ dysfunction and other TRTs were defined and graded as per the Common Terminology Criteria for Adverse Events Version 5.[6] Proportion of children developing toxicity and toxicity profile were described. The possible risk factors for TRT and mortality were analyzed.

Statistical Analysis

Data analysis was performed using SPSS software version 22.0. Categorical variables were presented as frequencies and percentages. The normality of continuous data was assessed using the Kolmogorov–Smirnov test. For normally distributed continuous variables, mean and standard deviation were used, while nonnormally distributed variables were expressed as median with interquartile range (IQR). Categorical variables were analyzed using the chi-square test or Fisher's exact test as appropriate.

To compare continuous data at the beginning and end of the study, a paired Student's *t*-test was applied for normally distributed data, whereas the Wilcoxon signed-rank test was used for nonnormal data. Univariate regression analysis was performed to assess the associations between $\geq$ grade 3 nonhematological toxicities and potential risk factors.

Results

Baseline Characteristics of the Study Population

A total of 112 children were eligible for enrollment in the study and 11 were excluded as they refused treatment or opted for care from another center. One hundred and one children were included in the study, of which 64 (63.37%) were male and 37 (36.63%) were female. Median age of the study population at diagnosis was 6 years (IQR 3, 11). One child (1%) was <1 year, 69 (68.32%) were between 1 and 10 years, and 31 (30.68%) were >10 years old. Median duration of complaints was 15 days (IQR 7, 30). Examination at admission revealed bulky lymphadenopathy in 7 (6.93%), bulky liver in 41 (40.59%), bulky spleen in 26 (25.74%), and bulky liver as well as spleen in 21 (20.79%) children. Mediastinal mass was observed in 17 (16.83%), pleural disease in 3 (2.97%), and central nervous system (CNS) involvement by clinical examination was observed in 3 children (2.97%). However, testicular involvement was observed in none and there was no radiologically or cytologically demonstrable CNS disease. Risk stratification was done based on the clinical and laboratory characteristics at admission, day 8 prednisolone response, and cytogenetics into standard risk, intermediate/medium risk, and high risk in 24 (23.76%), 29 (28.71%), and 48 (47.52%) children, respectively.

Thirty-one children (30.69%) had total leukocyte count (TLC) of $>50\times10^3/\mu L$ at presentation. Among the bone marrow/peripheral blood cytogenetics analyzed, favorable cytogenetics was observed in 21 children (20.82%) and high-risk cytogenetics was observed in 7 (6.9%) children, which included *t*(9;22) in 5 (4.9%), and Mixed-lineage leukemia (MLL) rearrangement in 2 (1.9%). Prednisolone response assessment on day 8 was done for 95 children, of which 20 children (21.05%) were prednisolone poor responders. Bone marrow assessment for MRD on day 35 was done for 81 children, of which 28 (34.56%) were MRD positive. Among the children with MRD positivity, the following observations were noted: 23 (82.1%) had B-lymphoblastic leukemia (B-ALL) and 5 (17.8%) had T lymphoblastic leukemia (T-ALL). Seven children each (50%) belonged to standard risk and intermediate risk, and 14 (50%) belonged to high risk at admission. The median duration of hospital stay of these patients was 40 days (36–43).

Flow cytometric analysis of the bone marrow/peripheral blood revealed B-ALL in 77 (76.24%), T-ALL including Early T-cell precursor (ETP)-ALL in 19 (18.81%), T-LL in 4 (3.96%) children, and mixed phenotype acute leukemia (B-lymphoblastic and myeloid leukemia) in 1 (0.99%) child.

The median *z*-scores for weight, height/length, and body mass index at admission were -1.53 (-2.12, -0.81), -0.99 (-1.65, -0.15), and -1.26 (-2.36, -0.45), respectively. Number of children who were underweight, stunted, and malnourished at admission were 30 (29.7%), 19 (18.8%), and 34 (33.7%), respectively.

Primary Objective

In the population studied, there were a total of 696 toxicities recorded among 101 children, of which 418 (60.06%) were of grade 3 and above, which amounts to a mean of 4 toxicities per person. Number of grades 3, 4, and 5 toxicities, in decreasing trend were 231 (33.1%), 180 (25.8%), and 7 (1%), respectively. Since the outcome of interest was toxicities of grade 3 and above, the profile of those toxicities was studied in detail. From the system-wise description of toxicity profile, it is observed that 73.2% were hematological and 26.8% were nonhematological. The proportion of TRTs is

Table 1 Systemwise description of toxicity profile (≧ grade 3)

System involved	Grades 3–5, *n* (%)	Grade 3, *n* (%)	Grade 4, *n* (%)	Grade 5, *n* (%)
Hematological	306 (73.2)	150 (49.02)	156 (50.98)	0 (0)
Infectious	27 (6.46)	17 (62.96)	5 (18.5)	5 (18.5)
Cardiovascular	26 (6.22)	24 (92.31)	2 (7.69)	0 (0)
Metabolic	22 (5.26)	13 (59.09)	7 (31.81)	2 (9.09)
Gastrointestinal	15 (3.59)	15 (100)	0 (0)	0 (0)
Renal	10 (2.39)	5 (50)	5 (50)	0 (0)
Hepatobiliary	7 (1.67)	5 (71.43)	2 (28.57)	0 (0)
Central nervous	5 (1.12)	2 (40)	3 (60)	0 (0)

summarized in ►**Table 1**. The description of toxicities affecting each organ system, including the individual toxicities observed, and their week-wise distribution during induction chemotherapy, is summarized in ►**Table 2**.

It is observed that majority of the hematological toxicities of higher grades occurred in week 1 as summarized in ►**Table 2**. A total of 59 febrile neutropenic events were recorded among 50 children. Of these, 42 children (84%) had B-ALL, 7 (14%) had T-ALL, and 1 (2%) had mixed phenotype. Based on clinical and laboratory criteria, these children were risk-stratified as follows: 7 (14%) in the standard-risk group, 19 (38%) in the intermediate-risk group, and 24 (48%) in the high-risk group. Nutritional assessment at admission revealed that 15 children (30%) were underweight, 10 (20%) were stunted, and 17 (34%) were wasted. Majority (32.2%) were observed in week 3, followed by week 2 (25.42%), and the least (5.08%) in week 5. The number of grade 3 and grade 4 febrile neutropenic events were 55 (93.2%) and 4 (6.8%), respectively. The median time to resolution of febrile neutropenic events was 3 days (IQR 1, 2).

Among the metabolic toxicities, tumor lysis syndrome (TLS) was the most common, seen in 17 children (73.9%), most of which occurred in week 1 (82.35%) with median time to resolution (for toxicities of grades 3 and 4) of 3 days (2–4). Of these, 9 children (52.9%) had B-ALL, 7 (41.1%) had T-ALL, and 1 (5.8%) had T-LL. These children were risk-stratified as follows: 1 (5.8%) in the standard-risk group, 3 (17.6%) in the intermediate-risk group, and 13 (76.4%) in the high-risk group. Nutritional assessment at admission revealed that 5 children (29.4%) were underweight, 2 (11.7%) were stunted, and 5 (29.4%) were wasted. TLS of grades 3, 4, and 5 were observed in 8 (47.06%), 7 (41.1%), and 2 (11.7%), respectively. Hyperglycemia was recorded in 3 children (13.1%), all older than 10 years, during weeks 1 and 2, treated with oral hypoglycemic agents and insulin, and median time to resolution noted was 15 days (12, 28). Hypertension was the only cardiovascular toxicity observed in the population studied with majority occurring in weeks 1 and 2 (30.77 and 34.61%, respectively) with median time to resolution of 14 days (10–20). The common gastrointestinal toxicities observed were diarrhea, chemo-induced nausea and vomiting (CINV), and oral mucositis, as described in ►**Table 2**, most of which occurred in week 2. The observed hepatoxicities include transaminitis and hyperbilirubinemia, which occurred in weeks 1 and 2.

The observed renal toxicities include acute kidney injury (AKI) in majority (80%), all of which are reported in week 1 and tubulopathy in the rest (20%), reported in weeks 3 and 4. According to the KDIGO (Kidney Disease: Improving Global Outcomes) criteria, stage 3 AKI was observed in 5 children (4.95%). There were 5 CNS toxicities reported; with seizures being the most common (40%) attributed to electrolyte imbalance.

Infectious Complications

Fifty-three infectious complications were observed in the cohort studied, which include both clinically and microbiologically documented infections. Of which 27 were of grade 3 and above, which include blood stream infections (BSIs) (55.5%), description of which is given in ►**Table 3**; soft tissue, gastrointestinal, and lower respiratory tract infections contribute to the rest (44.5%). The median time to resolution of BSI (of grades 3 and 4) was 10 days (8–14). The incidence of clinically documented infections (CDIs), microbiologically documented infections (MDI), BSI, and multiorgan dysfunction syndrome (MODS) is presented in ►**Table 3**. Culture-proven BSI were documented in 15 children (14.85%), of which 9 (8.91%) went into MODS/septic shock.

Secondary Objectives

A total of 9 mortalities were observed among the population studied (8.9%). The causes of mortality are listed in ►**Table 4**. Infection-related mortality was observed in 5 children (55.5%). Risk factors associated with mortality could not be analyzed in this study as the event rate was less and hence could not be compared with the rest of the population studied. However, among this population, majority (77.8%) belonged to the high risk, 44.4% expired in week 1 of illness, and one had Downs phenotype.

Out of the 101 children studied, treatment delay was observed in 51 children (50.49%) during the study period; of which 37 (72.5%) had single treatment break and 14 (27.45%) had treatment breaks in two separate occasions. Hence, a total of 65 treatment breaks were observed and the total number of days in breaks was 294, which accounts for a mean

Table 2 Profile of aforementioned toxicities

	Toxicity	Number of toxicities analyzed *n* (%)	Week 1 *n* (%)	Week 2 *n* (%)	Week 3 *n* (%)	Week 4 *n* (%)	Week 5 *n* (%)
1	Hematological ($n = 306$)						
	Anemia	84 (27.45)	74 (88.1)	5 (5.95)	2 (2.38)	3 (3.57)	0 (0)
	Neutropenia	85 (27.78)	24 (28.23)	16 (18.82)	41 (48.23)	4 (4.72)	0 (0)
	Thrombocytopenia	78 (25.49)	53 (67.95)	13 (16.67)	11 (14.1)	1 (1.28)	0 (0)
	Febrile neutropenia	59 (19.28)	13 (22.04)	15 (25.42)	19 (32.20)	9 (15.25)	3 (5.08)
2	Infectious ($n = 15$)						
	BSI	15	3 (20)	3 (20)	4 (26.6)	4 (26.6)	1 (6.7)
3	Metabolic ($n = 22$)						
	TLS	17 (73.9)	14 (82.35)	3 (17.65)	0 (0)	0 (0)	0 (0)
	Hyperglycemia	3 (13.1)	2 (66.67)	1 (33.33)	0 (0)	0 (0)	0 (0)
	Hypocalcemia	1 (8.7)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)
	Hypokalemia	1 (4.3)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)
4	Cardiovascular ($n = 26$)						
	Hypertension	26	8 (30.77)	9 (34.61)	5 (19.23)	4 (15.39)	0 (0)
5	Gastrointestinal ($n = 22$)						
	Diarrhea	4 (18.18)	0 (0)	2 (50)	0 (0)	2 (50)	0 (0)
	Transaminitis	4 (18.18)	2 (50)	2 (50)	0 (0)	0 (0)	0 (0)
	Hyperbilirubinemia	3 (13.63)	1 (33.33)	2 (66.67)	0 (0)	0 (0)	0 (0)
	CINV	3 (13.64)	1 (33.33)	2 (66.67)	0 (0)	0 (0)	0 (0)
	Oral mucositis	3 (13.63)	0 (0)	1 (33.33)	1 (33.33)	1 (33.33)	0 (0)
	Ileus	2 (9.09)	0 (0)	1 (50)	1 (50)	0 (0)	0 (0)
	Pancreatitis	2 (9.09)	0 (0)	1 (50)	1 (50)	0 (0)	0 (0)
	Gastritis	1 (4.54)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
6	Renal ($n = 10$)						
	AKI	8 (80)	8 (100)	0 (0)	0 (0)	0 (0)	0 (0)
	Tubulopathy	2 (20)	0 (0)	0 (0)	1 (50)	1 (50)	0 (0)
7	Central nervous system ($n = 5$)						
	Seizure	2 (40)	1 (50)	1 (50)	0 (0)	0 (0)	0 (0)
	PRES	1 (20)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
	Leukoencephalopathy	1 (20)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
	Traumatic myelopathy	1 (20)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)

Abbreviations: AKI, acute kidney injury; BSI, blood stream infections; CINV, chemotherapy-induced nausea and vomiting; PRES, posterior reversible encephalopathy syndrome; TLS, tumor lysis syndrome.

delay of 5.76 days per person. Maximum number of treatment interruptions were observed in week 3 (33.9%), followed by week 2 (27.7%), week 4 (20%), week 1 (16.9%), and week 5 (1.5%). It is observed that majority of treatment interruptions (75.38%) were due to febrile neutropenia (44.6%) and infection-related (CDI 15.38% and MDI 15.38%). The rest were due to hepatotoxicity (7.69%), CINV (4.61%), suspected pancreatitis (6.15%), ileus (4.61%), and seizures (1.53%). Treatment/protocol modification was observed in 10 (9.9%); of which protocol modification was observed in 4 (36.3%) children (high risk to intermediate risk in 3 and to standard risk in 1); chemotherapeutic agent dose reduction was seen in 5 children (49.5%), both owing to excessive toxicities, and alternate chemotherapeutic agent was used in 1 child (9.2%) with CNS metastasis.

A statistically significant association was observed between risk group and nonhematological toxicities of grade $\geq$ 3, with the nonstandard risk group (intermediate- and high-risk groups) exhibiting 3.21 times higher odds of experiencing these toxicities compared with the standard-risk

Table 3 Incidence of CDI, MDI, and sepsis

S. no.	Infections	$N = 101$ *n* (%)
1	**CDI**[a]	**24 (23.76)**
	• Upper respiratory tract infections	9 (37.5)
	• Gastrointestinal infections	7 (29.2)
	• Oral infections	4 (16.6)
	• Lower respiratory tract infections	3 (12.5)
	• Soft tissue infection	1 (4.2)
2	**MDI**[b]	**29 (28.7)**
	• **Blood stream infections**	**15 (51.7)**
	Bacterial	
	MSSA[c]	3 (20)
	Pseudomonas aeruginosa	2 (13.33)
	Escherichia coli	2 (13.33)
	Salmonella enterica	2 (13.33)
	Klebsiella pneumoniae	1 (6.66)
	MRSA[d]	1 (6.66)
	Streptococcus dysgalactiae	1 (6.66)
	Fungal	
	Candida albicans	2 (13.33)
	Trichosporon azaki	1 (6.66)
	• **Upper respiratory tract infections**	**8 (27.58)**
	RSV[e]	3 (37.5)
	Influenza	5 (62.5)
	• LRI[f]- Pulmonary aspergillosis	2 (6.89)
	• **Soft tissue infections**	**2 (6.89)**
	MSSA[c]	1 (50)
	MRSA[d]	1 (50)
	• UTI[g] (Klebsiella)	1 (3.44)
	• Otitis media (Pseudomonas aeruginosa)	1 (3.44)
3	**Septic shock/MODS**[h]	**9 (8.91)**

[a] CDI, Clinically documented infection; [b] MDI, Microbiologically documented infection; [c] MSSA, Methicillin sensitive Staphylococcus aureus; [d] MRSA, Methicillin resistant Staphylococcus aureus; [e] RSV, Respiratory syncytial virus; [f] LRI, lower respiratory tract infection; [g] UTI, urinary tract infection; [h] MODS, Multiorgan dysfunction syndrome.
Note: Boldfaced values indicate the proportion of the main group, with the corresponding subgroup proportions presented in the same cell.

group with a *p*-value of 0.022 (95% confidence interval [CI] 1.18–8.71) (►**Table 5**). As only one factor was found to be statistically significant, multivariable regression analysis was not performed.

Discussion

In this observational cohort study, we prospectively analyzed TRTs of ≥ grade 3 in children aged 1 month to 15 years with ALL/T-LL undergoing induction chemotherapy. We reported their incidence, profile and timing, assessed risk factors associated with these TRT, along with treatment delays, and outcomes in terms of mortality and MRD status.

Toxicities

Among the toxicities observed, 60% were ≥ grade 3 and only those were analyzed due to clinical relevance. The incidence of grade 3, 4, and 5 toxicities was 33.91%, 25%, and 1%, respectively, compared with 32.1%, 16%, and 4.5% in another study.[7] The most common toxicity observed was hematological (73.2%), significantly higher than the 45.8% reported in a Mexican study.[7] A total of 169 nonhematological toxicities ≥ grade 3 were observed in 77 children (mean: 2.19 toxicities/person). Several studies report 40% of ALL children experiencing ≥ 1 TRT.[8]

Hematological Toxicities

Anemia, neutropenia, and thrombocytopenia ≥ grade 3 occurred in 27.45%, 27.78%, and 25.49%, respectively, of our study population; Ozdemir et al reported higher incidence of 34%, 51% (≧ grade 3 anemia and thrombocytopenia, respectively), and 60% (grade 4 neutropenia).[9] Febrile neutropenia accounted for 19.28% of hematological toxicities and 14.11% of all ≥ grade 3 toxicities.

Nonhematological Toxicities

In a retrospective analysis done by Zawitkowska et al on grade 3 and 4 toxicities, the common nonhematological toxicities reported were infectious in 32.3%, hepatic in 28.2%, gastrointestinal in 20.64%, followed by the less

Table 4 Risk factors and causes of mortality

S. no.	Age (y)	Gender	Risk group	Immunophenotype	Day of outcome	Cause of mortality
1	14	Male	HR	T-ALL	23	Septic shock
2	11	Male	HR	B-ALL	6	TLS- hyperkalemia
3	4	Male	HR	T-ALL	6	Intracranial hemorrhage
4	13	Female	IR	B-ALL	35	Septic shock
5	15	Male	HR	B-ALL	7	Septic shock
6	4	Female	HR	B-ALL	20	Septic shock
7	13	Male	HR	T-ALL	8	CNS metastasis- Raised ICP
8	5	Female	IR	B-ALL	8	Septic shock
9	13	Male	HR	B-ALL	7	TLS- AKI

Abbreviations: AKI, acute kidney injury; B-ALL, B-lymphoblastic leukemia; CNS, central nervous system; HR, high risk; ICP, intracranial pressure; IR, intermediate risk; T-ALL, T-lymphoblastic leukemia; TLS, tumor lysis syndrome.

Table 5 Univariate analysis of the risk factors associated with nonhematological toxicities of grades 3 and above among the study population

Characteristics	Total	Toxicity present *n* (%)	No toxicity *n* (%)	Unadjusted risk ratio (95% CI)	*p*-Value
Age					
1–10 y	69	52 (75.40)	17 (24.6)	–	0.76
< 1 y or > 10 y	32	25 (78.1)	7 (21.9)	1.16 (0.43–3.17)	
Gender					
Male	64	47 (73)	17 (27)	–	0.387
Female	37	30 (81.1)	7 (18.9)	1.55 (0.57–4.18)	
Risk group					
Standard risk	24	14 (58.3)	10 (41.7)	–	0.022
Nonstandard risk	77	63 (81.8)	14 (18.2)	3.21 (1.18–8.71)	
Protocol followed					
ICiCLe	84	65 (77.4)	19 (22.6)	–	0.55
BFM-95	17	12 (70.6)	5 (29.4)	0.71 (0.22–2.24)	
Flow cytometry					
B-ALL	78	62 (79.5)	16 (20.5)	–	0.163
T-ALL	23	15 (65.2)	8 (34.8)	0.48 (0.17–1.34)	
Underweight and severe underweight at admission (< 2 *z*-score)					
No	71	54 (76.1)	17 (23.9)	–	0.947
Yes	30	23 (76.7)	7 (23.3)	1.03 (0.37–2.83)	
Stunting and severe stunting at admission (< 2 *z*-score)					
No	84	66 (78.6)	18 (21.4)	–	0.226
Yes	17	11 (64.7)	6 (35.3)	0.5 (0.16–1.53)	
Thinness and severe thinness at admission (< 2 *z*-score)					
No	67	51 (76.1)	16 (23.9)	–	0.969
Yes	34	26 (76.5)	8 (23.5)	1.02 (0.38–2.69)	

Abbreviations: B-ALL, B-lymphoblastic leukemia; BFM-95, Berlin-Frankfurt-Munster-95; CI, confidence interval; ICiCLe, Indian Collaborative Childhood Leukemia group; T-ALL, T-lymphoblastic leukemia.

common toxicities, CNS, cardiac, and renal and skeletal in 2.9%, 2.7%, and 1.5%, respectively.[5] Studies by Stary et al and Demidowicz et al also reported similar system-wise toxicities.[10,11] A Mexican study reported induction chemotherapy toxicities in 81.2% of children (mean: 2 toxicities/person), with febrile neutropenia (18.8%), allergic reactions (6.3%), and sepsis (6%) being common.[7] In our study, infections were predominant among nonhematological toxicities, but gastrointestinal and hepatic toxicities were substantially lower.

Infections comprised 6.46% of all toxicities, with BSI being the most common (14.85%). Reported infection rates in other studies range from 25% to 58%.[7,12] In a study done in Taiwan, fever was reported to occur at any point in 86.9%, febrile neutropenia was observed in 64%, CDI in 39%, and MDI in 44%.[13] The magnitude of infectious toxicities in other studies was substantially higher compared with our study, which could probably be due to differences in the characteristics of the study population, protocols used, and with those being studied at different phases of chemotherapy.

Our study reported febrile neutropenia in 19% and MODS/septic shock in 9%, lower than the 10% to 19% reported in another South Indian study by Rajeswari et al.[14] BSIs were mostly monomicrobial, unlike Brazilian studies reporting polymicrobial infections (6%). Our fungal sepsis rate (20%) was considerably higher than the reported 4.7%.[15]

Gastrointestinal toxicities (≥ grade 3) occurred in 3.59% of our study population, including diarrhea (0.9%), mucositis (0.7%), CINV (0.7%), and pancreatitis (0.5%). Literature reports higher rates of gastrointestinal toxicities accounting for 20.4%[5]; with mucositis (5%), CINV and, diarrhea (6%) during induction,[7] and pancreatitis (5–10%).[16] Age > 10 years, genetic predisposition, and presence of hypertriglyceridemia were observed risk factors for pancreatitis by Hough and Vora.[17] Hepatotoxicity was observed in 1.67%, significantly lower than reported rates of 15.6 to 28%.[18,19]

Hyperglycemia (≥ grade 3) was seen in 2.97% of cases, lower than the 10% to 20% reported in literature.[20] TLS occurred in 16.8% of cases, with clinical TLS in 8.9%, significantly lower than the reported 45% to 62%.[21] Hyperuricemia and hyperphosphatemia were the most common biochemical abnormalities reported in our study consistent with the literature.[21] Lower incidence in our study could be due to prophylactic measures including aggressive hydration, use of xanthine oxidase inhibitor (allopurinol) and better monitoring. Our study found hypertension in 6.2% of cases, lower than literature-reported rates (13–17.8%).[19] Renal toxicity was observed in 2.39%, with AKI in 7.9% and tubulopathy in 2%, lower than literature-reported 14% and 12.5%, respectively.[19,21]

Neurological toxicities were rare (1.12%), including seizures, posterior reversible encephalopathy syndrome, and myelopathy. Seizure incidence (2%) was much lower than the reported 10% to 15%.[19] Literature reports neurotoxicity in 3.6%, mostly during induction, including methotrexate-induced leukoencephalopathy (35.4%) and vincristine-induced vocal cord paralysis (14%).[22] We observed one case of methotrexate-induced leukoencephalopathy and one of traumatic myelopathy post-intrathecal methotrexate.

Treatment Delays

TRTs often led to treatment modifications and delays, potentially impacting treatment efficacy. In our study, delays were observed in 50.49% of children. Yeoh et al reported neutropenia (33%), febrile neutropenia (19%), and severe infections (19%) as the reasons for treatment delay, which is on par with our study.[23] Li et al reported delays due to infections in 7% of children, with a higher median duration of 8 days.[24] Ozdemir et al found that delays before day 8 increased mortality risk by 30%, especially in high-risk groups.[9] In our study, 16.9% of breaks occurred in the first week, peaking to 33% in week 3. Hence, attempts should be made to reduce treatment breaks anticipating toxicities during mid-weeks of induction.

Among 51 (50.4%) children with treatment delay, induction outcome in terms of MRD status are as follows: 37 (36.6%) were MRD negative, 9 (8.9%) were positive, and analysis could not be done in 5 children (4.9%) ►**Supplementary Table S1** (available in the online version only). These findings suggest that short-term treatment delays during induction may not uniformly lead to poor MRD outcomes and could be influenced by underlying disease biology, early response to therapy, and these results could possibly be due to selection bias/confounding factors resulting in skewing. However, the unexpected MRD response in children with treatment delays warrants further investigation through larger, prospective studies.

Mortality

Induction mortality in developed countries ranges from 1% to 2.8%,[10,25–27] with infections (72%) and bleeding/thrombosis (9%) as leading causes.[16] In developing countries, TRM ranges from 10% to 29% with majority due to infections and bleeding.[2,28,29] Mortality outcomes from our study reveal that the incidence of induction mortality tends to be higher compared with developed countries and lower compared with developing countries; however, the causation seems to be comparable. These findings highlight the need for enhanced supportive care, early initiation of antimicrobial therapy, guideline-based platelet transfusions, and improved health care accessibility. Predictive risk factors for induction mortality include female gender, Down syndrome, high-risk disease (odds ratio [OR] 1.84), high initial TLC (OR 1.02), low initial platelet count, and long travel time to health care facilities (OR 1.06/hour).[16,30] However, due to a low event rate, these associations could not be analyzed in our study.

Risk Factors for Toxicities

Both genetic and acquired factors influence TRM and individual toxicities. Studies highlight age, gender, immunophenotype, risk group, chemotherapy intensity, and day 33 remission status as significant factors. Older age and T-ALL are independent risk factors for toxicities, with incidence increasing from 44.5% (1–9 years) to 57.6% (10–14 years).[25] Older children have higher risks of developing pancreatitis, hepatotoxicity, hyperglycemia, and methotrexate-induced neutrotoxicity.[16] Meeske et al found female gender to be associated with increased grades 3 to 5 toxicities, treatment delays, infections, increased hospital stay, and supportive care requirements, as well as higher TRM risk.[31] Nonstandard risk groups had significantly higher toxicity risk (OR 3.21 [95% CI: 1.18–8.71], $p = 0.022$) in our study. However, no associations were found between age, gender, immunophenotype, and increased toxicity risk.

This prospective study provides valuable real-time data on TRTs during induction chemotherapy in children with ALL/T-LL in a resource-limited setting, using standardized criteria. This study offers granular, week-wise data on both hematological and nonhematological toxicities, highlighting peak periods and duration of specific toxicities, that can guide targeted supportive care. This single-center design limits generalizability and as this was limited to the induction phase, long-term and cumulative toxicities could not be assessed. While these results are particularly relevant to similar low- and middle-income settings, a more detailed subanalysis of specific toxicities stratified by each risk factor would indeed offer additional insights. Future multicenter studies with longer follow-up, evaluation of nutritional status, and supportive interventions are needed to better understand and mitigate these toxicities, and should focus on optimizing supportive care interventions and identifying biomarkers predictive of severe toxicities to further improve outcomes in pediatric ALL.

Conclusion

Our study provides critical insights into TRTs during induction chemotherapy in pediatric ALL. While hematological toxicities were predominant, infectious complications, though lower than in other studies, remained a notable concern. Treatment delays were frequent, peaking in week 3. Mortality rates were lower than in developing countries but higher than in developed nations, emphasizing the need

for improved supportive care. High-risk disease was associated with increased toxicity, reinforcing the importance of personalized risk stratification and proactive management strategies. Future multicenter research with long-term follow-up should aim to evaluate nutritional status, refine supportive care measures, and identify early predictors of severe toxicities to improve treatment outcomes in children with ALL.

Authors' Contributions

M.S. and J.G.R. conceptualized the study, collected data, analyzed data, and prepared the manuscript. J.G.R. supervised the study, critically revised the manuscript, and act as a guarantor of the paper. The manuscript has been read and approved by all the authors and the requirements for authorship have been met. Each author believes that the manuscript represents honest work, and the information is provided in original form.

Ethical Approval

The study was approved by the Institutional Ethics Committee and the approval certificate numbered JIP/IEC/2022/151 dated 13.07.2022 was issued. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki (2013).

Patient Consent

Written informed consent was obtained from the parents or legally acceptable representatives of the participants and assent was obtained from the participants wherever applicable.

Conflict of Interest

None declared.

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FOXO1 Rearranged Alveolar Rhabdomyosarcoma with Aberrant Strong and Diffuse NKX2.2 Expression: Diagnostic Pitfall with Distinct Therapeutic Implication

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A 5-year-old male child presented with a gradually progressive mass lesion over the left shoulder region for the past 2 weeks. On local examination, there was a 4×5 cm mass over the anterior aspect of the shoulder, fixed to the deltoid muscle, as seen on the magnetic resonance imaging scan (►**Fig. 1**). The patient underwent a needle biopsy, and histopathology showed a malignant small round cell tumor (MSRCT). On the first immunohistochemistry (IHC) panel, the tumor cells were diffusely positive for NKX2.2, CD99, Desmin, Myogenin, and MyoD1, while being negative for Pancytokeratin, LCA, Synaptophysin, and NKX3.1 (►**Fig. 2**). In view of the ambiguous IHC findings, additional tests, including MUC-4 and ALK-1 (►**Fig. 3**), were performed. Both showed diffuse positive expression, and a final diagnosis of

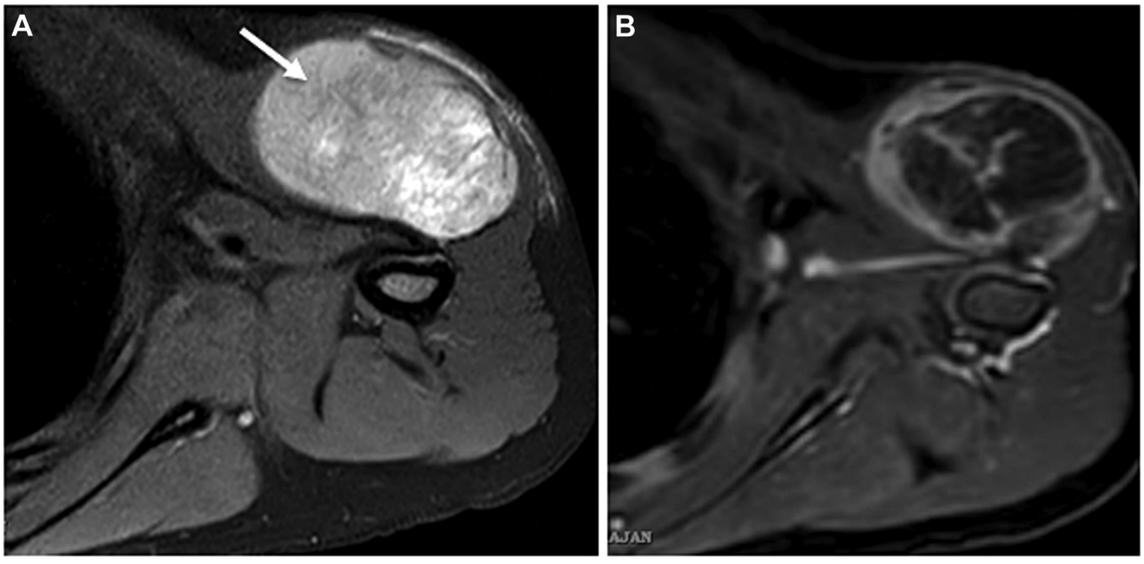

Fig. 1 T2-weighted MRI showing a hyperintense soft tissue mass in the left shoulder with heterogeneous enhancement, involving the deltoid muscle (*arrow*). No erosion of underlying bone seen.

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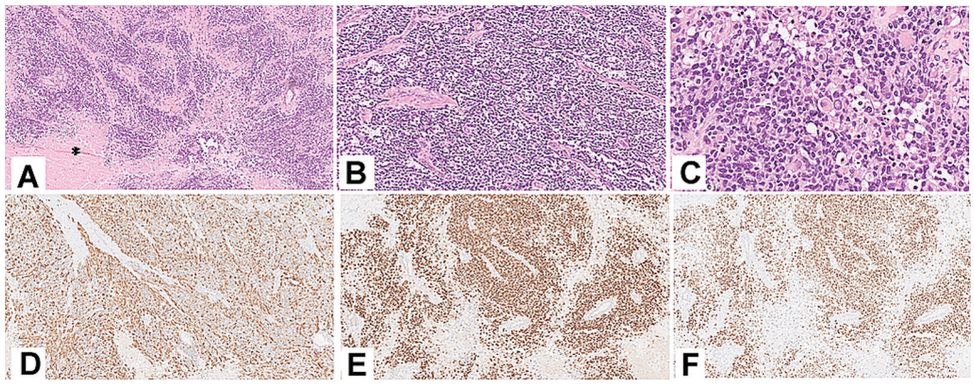

Fig. 2 (**A–C**) (10×, 20× 40×, H&E stain) Histopathology images showing a round cell tumor with extensive necrosis (*). (**D**–**F**) (20×, DAB stain) Immunohistochemistry images showing diffuse positivity for Desmin, Myogenin, and MyoD1, respectively.

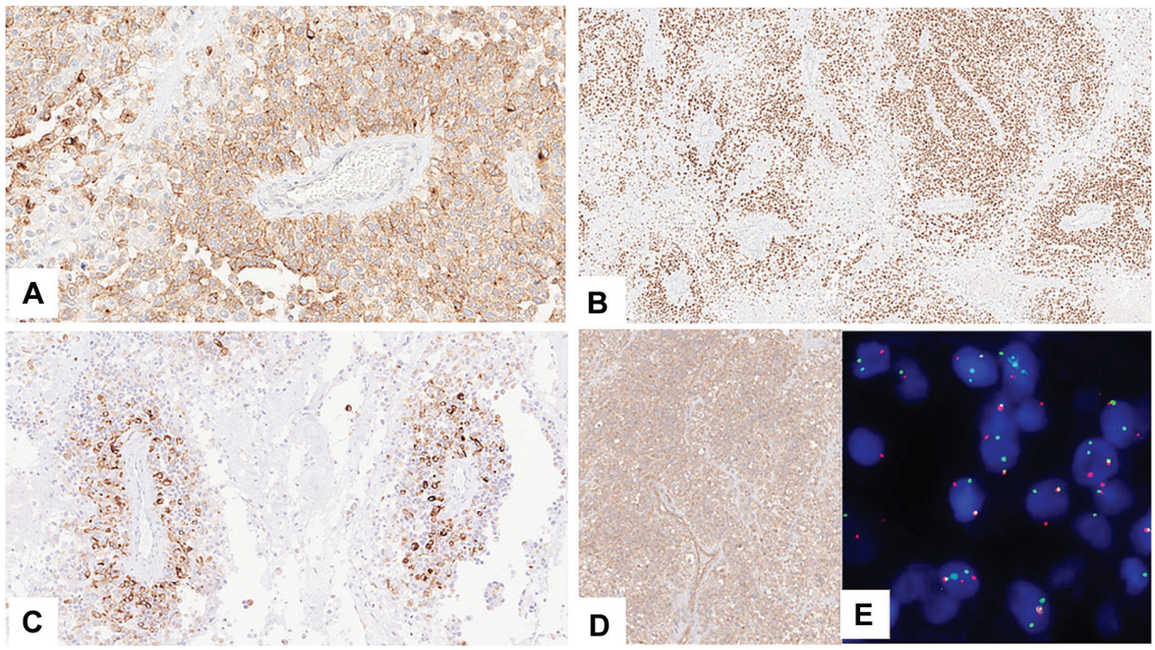

Fig. 3 (**A**) (20×, DAB stain): Tumor cells show diffuse and strong immunoreactivity for ALK-1 on immunohistochemistry. (**B**) (10×, DAB stain): immunohistochemistry images showing strong and diffuse NKX2.2 positivity and (**C**) MUC4 expression (**D**) while being negative for CD 99. (**E**) FOXO1 rearrangement seen on BA FISH.

Rhabdomyosarcoma (RMS) was rendered. Subsequently, break-apart fluorescence in situ hybridization (BA-FISH) revealed *FOXO1* gene rearrangement in 75% of the tumor cells, clinching the diagnosis of Alveolar RMS (fusion positive) with aberrant diffuse NKX2.2 immunoexpression.

The case was discussed in the multispecialty tumor board meeting and in view of no underlying bone involvement, a wide local excision was performed, followed by adjuvant chemotherapy with vincristine, actinomycin-D, cyclophosphamide (VAC) alternating with vincristine, irinotecan. After four cycles of chemotherapy, radiotherapy to the tumor bed was administered. Currently the child is receiving the remaining adjuvant chemotherapy and remains disease free.

NKX2.2 is an established IHC marker that is highly sensitive and specific for Ewing sarcoma (ES), aiding in the diagnostic workup of MSRCTs.[1,2] Alveolar RMS (ARMS) is an MSRCT with skeletal muscle differentiation, characterized by the presence of *PAX3-FOXO1* or *PAX7-FOXO1* gene fusions. Both of these entities have a similar clinical presentation, occurring in the deep soft tissues of children and young adults, but they differ widely in their therapeutic management. While ES is managed by neoadjuvant chemotherapy followed by surgery, RMS is treated with upfront surgery, reiterating the importance of an accurate diagnosis. On IHC, ARMS is immunopositive for Desmin, Myogenin, and MyoD1, while characteristically negative for NKX2.2. However, a few other MSRCTs can also show rhabdomyoblastic differentiation, as evidenced by positive myogenic markers, such as mesenchymal chondrosarcoma, which is also known to express NKX2.2.[3]

This makes additional IHC workup imperative when encountering an equivocal immunoprofile, as seen in this case. Recently, MUC4 has been described as a useful adjunct marker for the diagnosis of Alveolar RMS (fusion-positive),[4] which was also used in this case. Although not targetable, cytoplasmic overexpression of ALK protein is known to occur in the vast majority of ARMSs, making it another useful marker in the diagnostic armamentarium.[5]

The presented case highlights the significance of applying comprehensive IHC panel when dealing with MSRCTs of bone and soft tissue. MUC4 is ostensibly a very useful IHC marker and can be explored in further studies as a surrogate for fusion-positive alveolar RMS and to distinguish it from the fusion-negative embryonal RMS, due to distinct prognostic and therapeutic implications—especially in resource-limited centers where specialized molecular techniques, such as BA-FISH or next generation sequencing, are not available.

Authors' Contributions

S.P. and P.C. performed the histological examination. S.J. was involved with the treatment and follow-up of the patient. R.O. was responsible for the radiological workup. S. P. and P.C. wrote the main manuscript text. All authors reviewed and approved the final manuscript.

Patient Consent

Patient consent has been taken.

Conflict of Interest

None declared.

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Gut Microbiome as a Predictive Biomarker for Febrile Neutropenia in Pediatric Acute Lymphoblastic Leukemia: A Clinical Integrative Framework

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Introduction

Febrile neutropenia (FN) is a common and potentially life-threatening complication mostly observed in pediatric patients undergoing chemotherapeutic regimen for the treatment of hematological malignancies, such as acute lymphoblastic leukemia (ALL).[1] It is characterized by a single oral temperature $\geq$ 38.3°C (101.0°F) or an oral temperature $\geq$ 38.0°C (100.4°F) sustained for over an hour or occurring twice within a 24-hour period. An absolute neutrophil count (ANC) $< 0.5 \times 10^9$/L or ANC $< 1.0 \times 10^9$/L is expected to decrease to $< 0.5 \times 10^9$/L over the subsequent 48 hours, accompanied by fever.[2,3] Despite standardized chemotherapy protocols, FN risk in the pediatric population remains heterogeneous, with current risk stratification relying on post-onset clinical features, limiting early intervention.[4–6] Recent reports link baseline gut microbial dysbiosis, marked by reduced microbial diversity and elevated Proteobacteria, to FN complications, suggesting their prospective role as an anticipatory biomarker, providing a valuable window for early risk stratification and targeted supportive care.[7–14]

Predictive Value of Gut Microbial Signatures

The gut microbiome population comprises a highly dynamic ecosystem that gets profoundly disrupted by multiple interventions—including chemotherapy, mucositis, dietary alterations, and differential antibiotic exposures.[7,8,15] Dysbiosis—marked by reduced microbial diversity (such as intraspecific or α diversity) and expansion of opportunistic taxa—has strong associations with increased risk to infections.

Some key findings from pediatric ALL and hematopoietic stem cell transplantation (HSCT) cohorts include:

- Loss of gut microbial diversity often precedes neutropenia correlating with FN incidence and duration.[1,7,11]
- Expansion of specific microbial taxa, such as *Enterococcus*, is linked to prolonged FN episodes and adverse outcomes.[8,11,16]
- In pediatric HSCT recipients, low α diversity and an enriched antimicrobial resistome (pooled collection of microbiome resistance genes) predicts bloodstream infections, thereby underscoring a microbiome-based predictive potential in highly immunocompromised patients.[7,9,17]

These combined data indicate toward microbiome metrics as early biomarkers of infection risk in immunocompromised pediatric patients, such as those undergoing chemotherapy for ALL, enabling clinicians to predict and potentially prevent conditions such as sepsis and FN before the onset of clinical symptoms.[10,18]

Proposed Framework for Clinical Integration

To translate the above insights into future clinical practice, we hereby propose a three-tiered, evidence-based framework for integrating longitudinal gut microbial assessment into supportive care for pediatric ALL.

Tier 1: Baseline Microbial Profiling

- Collecting stool samples at diagnosis.
- Employing 16S rRNA sequencing to estimate α diversity and relative abundance of key gut microbial phyla.

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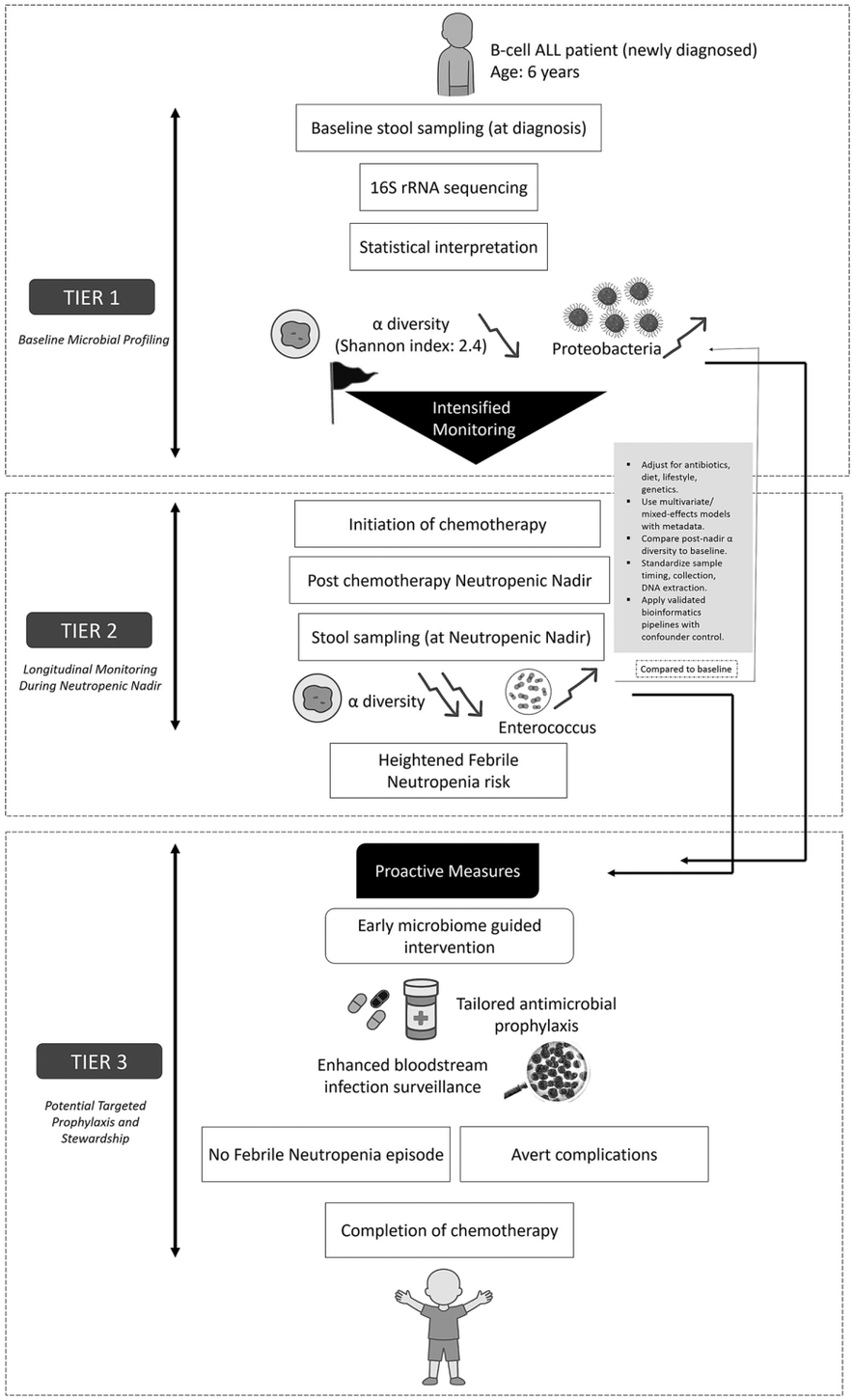

Fig. 1 Hypothetical application of the three-tiered gut microbiome framework in a pediatric ALL patient. A 6-year-old child with newly diagnosed B-cell ALL undergoes baseline stool sampling (Tier 1), revealing low α diversity (Shannon index 2.4) and increased Proteobacteria abundance, prompting flagging for intensified monitoring. During induction chemotherapy, stool analysis at neutropenic nadir (Tier 2) demonstrates further decline in microbial diversity and *Enterococcus* overgrowth. Based on these findings, Tier 3 interventions including tailored antimicrobial prophylaxis and enhanced surveillance for bloodstream infections are implemented. The patient completes chemotherapy without febrile neutropenia episodes, illustrating how early, microbiome-guided interventions may potentially prevent infectious complications. ALL, acute lymphoblastic leukemia.

- Linking alteration gut microbial diversity or relative abundance of potentially pathogenic taxa (e.g., Proteobacteria) to identify patients at elevated FN risk, thereby guiding intensified monitoring.[1,7]

Note: While Tier 1 enables early gut microbial risk stratification, it is critical to note that universally validated α diversity range or precise pathogenic taxa in pediatric ALL are not currently available. This lack of standardization stems from heterogeneity in sequencing platforms, analytical pipelines, and patient cohorts. Nevertheless, multiple studies, including Oldenburg et al (2021),[19] have consistently demonstrated that reduced microbial diversity and enrichment of taxa such as Proteobacteria or Enterococcaceae correlate with heightened infection risk. Specifically, a study by Hakim et al (2018) reported that a Proteobacteria relative abundance $\geq$ 0.01% or Enterococcaceae dominance $\geq$ 30% was associated with FN and infectious complications.[1] These findings further reinforce the clinical relevance of Tier 1 profiling, while emphasizing that such thresholds remain cohort-specific observations rather than validated diagnostic cutoffs.

Tier 2: Longitudinal Monitoring during Neutropenic Nadir (Lowest Count of Patient Neutrophil)

- Collecting stool samples at postchemotherapy neutropenic nadir using standardized timing and protocols to minimize preanalytical variability.
- Comparing postnadir α diversity alterations (if any) to baseline for predicting heightened FN risk, prompting earlier clinical interventions.[3,6]
- Ensuring reproducibility through consistent DNA extraction methods, centralized 16S rRNA sequencing pipelines, and validated bioinformatics workflows with stringent statistical adjustments for confounders.

Tier 3: Potential Targeted Prophylaxis and Stewardship

For identified high-risk FN patients from Tiers 1 and 2, potential proactive measures may include:

- Tailored antimicrobial prophylaxis preserving commensal bacteria.
- Targeted surveillance for bloodstream pathogens associated with dysbiosis.[9,10]

This approach can target antibiotic-driven gut dysbiosis, which contributes to infection susceptibility during neutropenic episodes, potentially mitigating FN and reducing hospitalization in pediatric patients.[15]

In developing the above three-tiered framework, it is essential to account for confounding variables such as antibiotic prophylaxis, geographical locations, dietary intake, genetic constitutions, lifestyle factors, and environmental exposures, which can substantially influence the gut microbial composition of patients.[20,21] Future analytic approaches should incorporate these metadata into statistical modeling or risk algorithms to ensure that predictions of FN risk reflect true microbial shifts rather than external modifiers. This strategy will enable more accurate, clinically meaningful interpretation of microbial profiling across diverse patient populations. A hypothetical application of the three-tiered system is provided in ►**Fig. 1**.

Translational Prospects and Future Implications

Although comprehensive metagenomic sequencing remains a cost-prohibitive approach for routine clinical practice, assessing simpler diversity indices or targeted resistome panels could offer practical and actionable insights.[7,11,22] Future prospective validation studies embedding longitudinal stool sampling with clinical outcomes are thus essential.[1,8,10]

As discussed earlier, it is important to recognize that gut microbial diversity and composition may vary profoundly across global populations due to multiple confounding factors.[20,21] Consequently, microbial signatures identified in one cohort may not be directly generalizable, underscoring the need for large multicentric validation across diverse populations before clinical implementation.

By standardizing and integrating gut microbiome assessment into clinical decision-making, FN management in pediatric ALL can shift from a reactive, clinical symptom–based approach to an anticipatory, biomarker-driven strategy; this in turn will potentially improve patient outcomes and reduce infection-related morbidity.[1–6,14,18,23]

Authors' Contributions

B.C. and R.D. collaboratively prepared and submitted the manuscript. B.C. has drafted the manuscript, and R.D. has proofread the manuscript and extensively helped by providing essential information.

Patient Consent

This is a Perspective article so patient consent is not required. No real patient, patient data or identifiable clinical information is involved. The illustrative case described is purely hypothetical and used for conceptual explanation only.

Conflict of Interest

None declared.

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Clinicians' Perceptions and Adoption of Oncology Biosimilars in India: Results from a National Survey

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Abstract

Biosimilars enhanced access to advanced therapies by providing cost-effective alternatives to reference biologics. However, their adoption in clinical practice remains limited due to concerns regarding efficacy, safety, and data reliability. This survey aimed to assess Indian clinicians' perspectives on biosimilars and identify factors influencing their adoption. A questionnaire-based online survey was conducted from July to August 2024. Fifty-two medical, radiation, and surgical oncologists across India provided a complete response. Questionnaires addressed prescription patterns, confidence in biosimilars, and clinical decision-making criteria. Descriptive statistics were performed. Among 52 respondents, 34.6% of clinicians prescribed biosimilars to >60% of patients, while 17.3% prescribed to <20%. Safety (27.1%), efficacy (35.6%), and pharmacokinetics (21.2%) were key determinants for biosimilar adoption; 76.9% of clinicians rejected biosimilars with biomolecular deviation; 65.4% of clinicians opposed data extrapolation without clinical trial evidence; and 76.9% lacked confidence in biosimilars before peer-reviewed publication. Clinicians stressed the importance of rigorous statistical analysis in biosimilar trials, with 46.2% hesitant without intention-to-treat/per-protocol analyses, 69.2% concerned about deviations beyond 80% to 125% margins, and 63.5% accepted efficacy differences within ± 20% of the noninferiority range. Clinicians' concerns and limited evidence hinder biosimilar adoption, despite their potential to improve access. Larger trials, transparent reporting, and real-world evidence may improve confidence.

Keywords
- biosimilars
- clinical practice
- survey
- oncology
- adoption

Introduction

Biosimilars are biologics that exhibit strong similarity to existing Food and Drug Administration (FDA)-approved reference drugs while offering a significantly more cost-effective alternative, making them particularly beneficial for resource-limited countries like India. The Biologics Price Competition and Innovation Act of 2010 introduced a simplified regulatory process to expedite their approval while ensuring they meet therapeutic standards.[1] India is emerging as a key player in the development and use of biosimilars, with several biosimilars already approved and in use for various cancer therapies.[2] However, the adoption rate has been relatively slow, mainly due to concerns about their safety and efficacy. Even after receiving regulatory approval, many clinicians are cautious about routinely incorporating biosimilars into clinical practice, particularly in oncology.[1,3]

Biosimilar development aims to demonstrate high similarity in structure, biological activity, efficacy, safety, and immunogenicity with the reference biologic. This allows the biosimilar to rely on the existing safety and efficacy data

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from the reference biologic, avoiding redundant clinical trials. Demonstrating biosimilarity involves comprehensive comparability studies. The extrapolation of biosimilar data across multiple indications continues to be a topic of ongoing discussion and scrutiny. While regulatory agencies allow extrapolation based on comprehensive comparability studies, concerns persist regarding its validity (including quality, nonclinical, and clinical data).[4] Literature suggests that although this approach is scientifically justified in many cases, it may not be universally applicable, particularly for complex biologics with multiple mechanisms of action. This study aimed to investigate the factors influencing biosimilar adoption among Indian clinicians and assess clinicians' prescription patterns, confidence in biosimilars, and decision-making criteria when considering biosimilars for cancer treatment.

Materials and Methods

A cross-sectional survey was conducted online over 2 months, from July to August 2024, to assess clinicians' perspectives on biosimilars and their adoption in oncology practice. The survey was designed based on insights from published literature on biosimilars and data regarding their availability in clinical practice. Questions were tailored and validated to address key aspects, such as familiarity with biosimilars, factors influencing prescription decisions, and perceived barriers to their use. The participants were recruited through professional networks, institutional contacts, and oncology-specific forums across India. The survey was conducted from July to August 2024. The survey questionnaire (►**Supplementary Table S1** [available in the online version only]) was distributed to practicing oncologists, including specialists in radiation, medical, and surgical oncology via professional network and email. A purposive sampling strategy was employed, targeting practicing oncologists relevant to the study's scope.

Descriptive statistics, primarily frequency counts and percentages, were used to analyze clinicians' responses and describe their prescribing patterns and attitudes toward biosimilars.

Ethical approval and informed consent were not required under the Common Rule, as this is a survey study and de-identified data were used.[5]

Results

A total of 76 oncologists were approached, of whom 52 responded, yielding a response rate of 68.4%. The final sample comprised 46 medical oncologists, 5 radiation oncologists, and 1 surgical oncologist. A summary table of key survey items and response distributions is given in ►**Supplementary Table S2** (available in the online version only). Significant variability in biosimilar adoption was observed among respondents. Notably, 34.6% ($n = 18$) of respondents reported that more than 60% of their patients received biosimilars, and 17.3% ($n = 9$) of clinicians indicated that biosimilars were prescribed to less than 20% of their patients. About 23% ($n = 12$) of clinicians prescribed biosimilars to 20% to 40% of their patients, while 25% ($n = 13$) prescribed them to 40% to 60% of their patients. Clinicians showed significant reluctance to consider biosimilars over innovator biologics. About 75% of respondents preferred not to use biosimilars and rather opted for biologics, while only 9.6% were open to adopting biosimilars and 15.4% were unsure.

Several clinical factors were found to influence the adoption of biosimilars in clinical practice among the clinicians who participated in this survey. A significant proportion of respondents (35.6%) identified similar efficacy as the most important factor, followed by similar safety (27.1%) and similar pharmacokinetic (PK) parameters (21.2%), similar to their reference standard. Only 16.1% of clinicians considered data from studies across all indications as a priority while prescribing biosimilars. Additionally, 45.3% of clinicians reported requiring a comprehensive evaluation across multiple efficacy endpoints before considering biosimilar use. Among individual parameters, 16.3% considered overall survival (OS), while 12.8% chose safety. Additional key factors included an overall response rate (ORR) of 9.3%, progression-free survival (PFS) of 8.1%, and PK parameters at 8.1% (►**Fig. 1**).

The presence of structural differences in biosimilars, such as oxidation or glycosylation issues, contributed to increased resistance among clinicians. A significant proportion of respondents (76.9%) rejected biosimilars with biomolecular deviations—defined as primary structural differences from the reference biologic—while only 13.5% were willing to proceed with their use. A majority of respondents (65.4%) opposed the extrapolation of biosimilar data across indications without supporting clinical trial data. 26.9% supported extrapolation, and 7.7% were unsure. Before peer-reviewed publication, the majority of clinicians (76.9%) lacked confidence in prescribing biosimilars, while 17.3% expressed comfort and 5.7% remained uncertain (►**Fig. 2**).

This apprehension extends to the design of clinical trials, with many clinicians emphasizing the importance of intention-to-treat (ITT) and per-protocol (PP) analyses. Most of the respondents (46.2%) were reluctant to consider a biosimilar study without ITT and PP analyses for clinical use. Additionally, 21.2% felt biosimilars could still be considered with modified intention-to-treat (mITT) without ITT and PP, and 32.6% were uncertain. About 69.2% of clinicians believed that deviations beyond the standard 80% to 125% margin would affect their clinical use of biosimilars. Only 21.2% disagreed, and 9.6% were uncertain. When asked about the impact of confidence intervals (CIs) at 90%, with efficacy parameters within $\pm 20\%$ of the predefined noninferiority range, a majority (63.5%) of clinicians responded positively. However, 21.1% were concerned and would refrain from adoption, while 15.4% remained uncertain (►**Fig. 2**).

Discussion

The results of this study underline the rigorous decision-making process regarding the adoption of biosimilars in oncology practice. While biosimilars hold the potential to

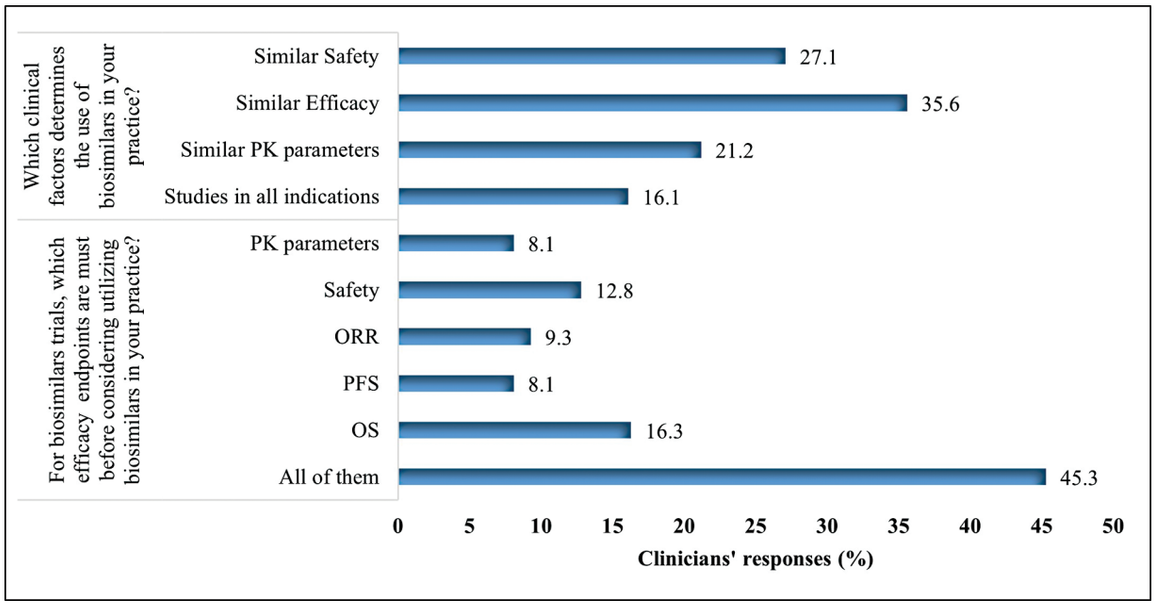

Fig. 1 Key factor considerations for integrating biosimilars into clinical practice.

reduce treatment costs and increase access to critical therapies,[1,3,6] the findings of this survey study indicate that clinicians remain cautious in their use due to concerns about clinical data consistency and the potential for differences in efficacy and safety. These findings are consistent with previous studies conducted in the United States and Europe demonstrating clinicians' prudence in biosimilar use,[7,8] especially when switching patients from bio-originator to biosimilar.[7] Furthermore, the clinicians rely on clinical studies and publications for evidence, as reported in previous studies.[9,10] A high proportion of clinicians reported requiring comprehensive safety and efficacy data across

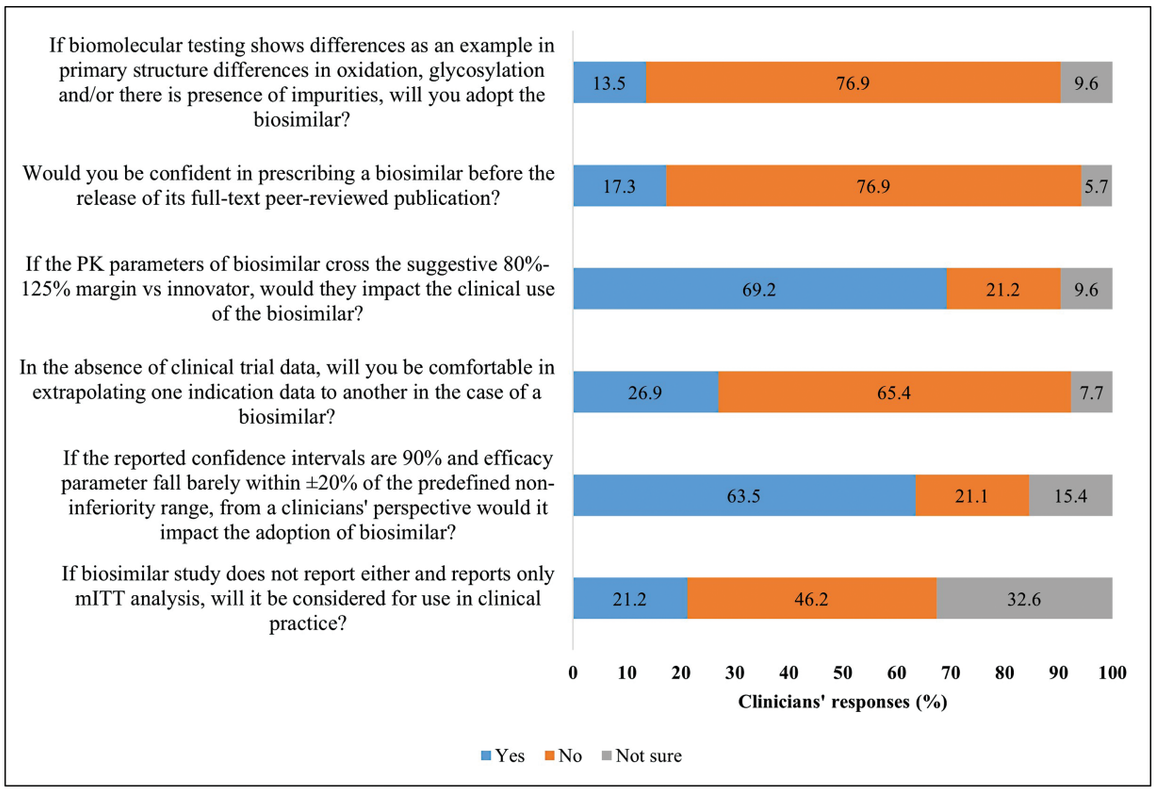

Fig. 2 Key factors influencing the adoption of biosimilars.

multiple endpoints, including OS, PFS, ORR, and PK parameters. This aligns with global oncology standards, where robust clinical evidence plays a crucial role in treatment decisions.[1,11] The majority of the clinicians in this survey emphasized the importance of ITT and PP analyses. The JAMA Guide to Statistics and Methods (2014) emphasizes that both ITT and PP analyses should be conducted and reported in a noninferiority trial,[12] highlighting the need for rigorous statistical reporting to maintain clinician confidence in clinical decision-making for biosimilars. Reluctance to accept biosimilars without peer-reviewed data and concerns about PK margins highlight the need for transparent, peer-reviewed clinical trial reporting.[13] This aligns with prior research suggesting that lack of long-term data and uncertainty about equivalence with reference products contribute to limited biosimilar use.[4] The importance of PK data is particularly evident,[4] as the majority of clinicians indicated that deviations beyond the acceptable margin would impact their prescribing behavior. While there is openness to biosimilars with a 90% CI within $\pm 20\%$ of the noninferiority range, concerns, uncertainty, and skepticism toward the extrapolation of untested indications highlight the need for further validation, transparency, and robust clinical trial evidence. These findings mirror the concerns demonstrated by the United States and European providers in terms of extrapolated indications, referring to the approval of biosimilars for indications held by the innovator biologic that were not directly assessed in the clinical trial of the biosimilar.[7] Despite India ranking first in approval of numerous biosimilars ($n = 98$) compared with the United States ($n = 26$) and European Union (EU, $n = 61$) in 2019, there remain concerns over regulatory rigor, pharmacovigilance system, clinical data, statistical validity, testing criteria in India compared with the U.S. and EU markets, which may have driven the skepticism demonstrated by the Indian clinicians toward biosimilars.[14,15] In contrast to Europe and America, India's fragmented regulatory framework, limited RWE dissemination, and lack of structured academic training on biosimilars may contribute to clinicians' hesitancy and less consistent adoption patterns.[16] Therefore, it is paramount to foster various industry–academic collaboration, rigorous post-marketing surveillance, and strengthen regulatory infrastructure, specifically quality control and approval processes, to boost confidence in biosimilars among clinicians.[15,17] It is also crucial to generate real-world evidence (RWE) to reinforce biosimilar safety and efficacy,[18] which could help validate biosimilar use in diverse patient populations. Clinicians need access to transparent, peer-reviewed studies demonstrating the safety and efficacy of biosimilars for a successful integration of biosimilars into oncology treatment regimens.

Limitations

One key limitation of this study is its reliance on self-reported data, which may introduce bias, particularly in the subjective reporting of prescribing practices and perceptions of biosimilars.[2] Second, the study was conducted among Indian clinicians, which may limit the generalizability of the findings to other regions with different regulatory frameworks and healthcare infrastructures. Moreover, the use of an online distribution method may have introduced selection bias, despite the efforts to disseminate the survey to a wider oncology community. Therefore, the participants may not accurately represent the broad cross-section of oncologists. Additionally, while the study identifies a lack of peer-reviewed data and RWE as key concerns influencing biosimilar adoption, it did not include a direct question comparing clinicians' preference for biologics versus biosimilars, which would have provided stronger support for the findings. Future studies are warranted with a larger sample representing a wide spectrum of healthcare professionals, to gain deeper insights on the real-world adoption of oncology biosimilars.

Conclusion

This survey highlights the multifaceted challenges in the adoption of oncology biosimilars in India. While biosimilars hold the potential to offer lower treatment costs and expand treatment choices, the lack of comprehensive, peer-reviewed clinical data, especially on efficacy and safety, raises significant concerns about biosimilar use among Indian clinicians. Therefore, clinicians remain cautious toward the adoption of biosimilars and emphasize the importance of transparent trial reporting and long-term data to boost confidence in biosimilars. To further enhance adoption, the study suggests the need for larger multicenter trials and RWE, and increased collaboration among different stakeholders to successfully integrate biosimilars into oncology practice.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' Contributions

The author confirms that they are the sole author of this article. The author meets the criteria for authorship as outlined in the journal's guidelines and affirms that the article represents honest and original work.

Ethical Approval

Ethical approval is not required under the Common Rule, as this is a survey study and de-identified data were used.

Patient Consent

Patient consent is not required.

Conflict of Interest

None declared.

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First Report of Variant *BCL6* Gene Rearrangement; Inversion 3: Clinical Presentation in a Rare Entity of Splenic B-Cell Lymphoma/Leukemia with Prominent Nucleoli

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Abstract

Splenic B cell lymphoma/leukemia with prominent nucleoli (SBLPN), previously known as hairy cell leukemia variant (HCL-V), remains a rare and provisional entity within the spectrum of splenic B cell neoplasms. It demonstrates overlapping morphologic and immunophenotypic features with low-grade B cell lymphomas, posing diagnostic and therapeutic challenges. We report the first case of inversion 3q27 causing a variant *BCL6* gene rearrangement within a complex clonal karyotype, characterized by multiple copy number alterations, that is, duplication 1q, deletion 7p, partial trisomy 8q, deletion 9q, deletion 10p, isochromosome 14q, trisomy 18, and trisomy X, indicative of marked genomic instability in a rare entity of SBLPN. The aggressive clinical course observed further emphasizes the need for comprehensive diagnostic workups for prognostication. As exact defining genetic alterations are not yet established for this entity, expanding the cytogenetic and molecular landscape is crucial for refining classification, prognostication, and therapeutic strategies. The case illustrates how genetic complexity may drive disease aggressiveness and management challenges, underscoring the need for early multidisciplinary interventions and individualized treatment strategies.

Keywords

- HCLv
- SBLPN
- complex karyotype
- chromosome 3q27 inversion
- variant *BCL6* gene rearrangement
- fluorescence *in situ* hybridization

Introduction

Hairy cell leukemia variant (HCL-V) is classified under the provisional category of splenic B cell lymphoma/leukemia with prominent nucleoli (SBLPN) in WHO-HAEM5. SBLPN is composed of medium to large mature B lymphoid cells with prominent nucleoli and is biologically distinct from both classical hairy cell leukemia (HCL) and other well-defined B cell non-Hodgkin lymphomas. Although it shares some morphologic and immunophenotypic features with HCL, it typically lacks CD5 expression, the BRAF V600E mutation, an aggressive clinical course, and resistance to standard HCL-directed therapies.[1]

Unlike other B-cell lymphomas, which often have well-defined genetic profiles, SBLPN lacks consistent prognostic genetic markers, largely due to its extreme rarity, estimated at just 0.03 cases per 100,000 individuals per year. Comprehensive genetic characterization is therefore essential for

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improving diagnostic accuracy, understanding disease biology, and refining prognostic assessment.

In HCL-V, limited studies have identified chromosomal abnormalities, most frequently involving complex karyotypes, 17p deletions, and various copy number alterations.[2,3]

To our knowledge, this is the first documented case of a complex karyotype in SBLPN featuring a novel pericentric inversion of chromosome 3;inv(3)(p13q27), involving a variant *BCL6* rearrangement (5′deletion) with 3p13 as the novel partner region, a finding not previously reported in this disease entity, additionally marked with unique copy number alterations.

We identified two divergent clonal populations: one harboring inv(3)(p13q27) leading to variant *BCL6* rearrangement with 5′ deletion, and the other clone showing a 3q deletion, resulting in *BCL6* loss. These findings reveal the underlying genetic heterogeneity of SBLPN and offer novel insights into the potential pathogenic role of *BCL6* alterations in its evolution and aggressive clinical behavior.

Case Presentation

A 50-year-old female presented with fever, severe pancytopenia, large hematomas, low Glasgow Coma Scale, and intracranial hemorrhage, requiring ICU admission. CT brain showed acute subdural hematomas in bilateral frontal, left temporal, and left parietal regions, with the largest (11 mm) extending into the posterior interhemispheric fissure.

At presentation, complete blood count showed Hb 8.5 g/dL, TLC 2.28 $\times 10^9$/L, PLT 03 $\times 10^9$/L, and ANC 0.75 $\times 10^9$/L; liver function tests revealed TB 23.2 µmol/L, DB 13.9 µmol/L, and ALT 60 U/L; and coagulation profile demonstrated PT 19 seconds, APTT 35 seconds, and INR 1.7. Peripheral smear findings showed severe pancytopenia with atypical lymphocytes and no circulating blasts (►**Fig. 1A**).

Bone marrow aspirate was hypocellular and hemodiluted, with approximately 80% infiltration by atypical small to medium-sized lymphoid cells exhibiting prominent nucleoli, cytoplasmic blebs, and occasional vacuoles (►**Fig. 1A–D**). Trephine biopsy showed hypercellular marrow with similar diffuse atypical lymphoid infiltration and normal reticulin fibers (Grade 0–1 MF; ►**Fig. 1C, D**).

Bone marrow flow cytometry demonstrated 65.5% lymphocytes, predominantly (70.3%) clonal CD19 +/CD20 +/sIgM +/lambda-restricted B-cells, negative for CD5, CD10, CD23, CD25, CD103, and other markers (►**Fig. 1E**).

The immunophenotype was inconsistent with CLL or mantle cell lymphoma, and supported a diagnosis of a B-lymphoproliferative neoplasm, favoring a hairy cell leukaemia variant, that is, SBLPN.

Karyotype and FISH Analysis

Karyotype analysis from unstimulated culture revealed a complex clonal abnormal karyotype with structural alterations involving chromosomes 1, 3, 7, 8, 9, 10, and 15, resulting in copy number changes and numerical abnormalities with gains of chromosomes 3, 14, 18, and X. Composite karyotype as per ISCN nomenclature[4] was designated as:

47∼49,XXX,dup(1)(q25q42),+der(3)inv(3)(p13q27),del(3)(q12),del(7)(p15p13),iso(8)(q10),der(9;15)(p10;q10)del(9)(p11),del(10)(p13),+14,iso(14)(q10),+18,+18[cp20] (►**Fig. 2A**).

Fluorescence in situ hybridization (FISH) was performed using probes for *MYC, BCL2, IGH/CCND1*, and *BCL6* genes (Cytocell, VYSIS, Downers Grove, Illinois, United States) that confirmed the karyotype findings.

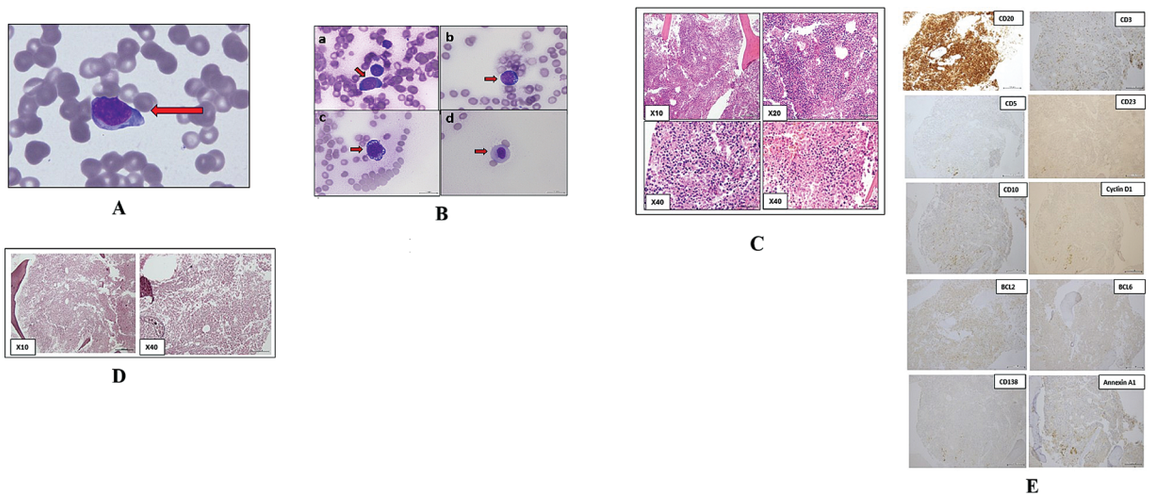

Fig. 1 (**A**) PB smear (×100). Lymphocytosis with many reactive and some atypical forms, the figure shows an atypical—spindle-shaped—lymphoid cell. (**B**) Bone marrow aspirate (Wright stain, ×100) BM with infiltrated approximately 80% atypical lymphoid cells; small to medium-sized with occasional nucleoli, cytoplasmic blebs/spindle shaped (a) and/or vacuolations (b, c), and some showed fried-egg like morphology (d). (**C**) Bone marrow trephine biopsy (H&E stain). (**D**) BM trephine biopsy reticulin special stained slides showing normal pattern of reticulin fibers (Grade 0–1 MF). (**E**) BM trephine biopsy immuno-stained slides showing strong diffuse positive CD20, partial positive BCL2 and annexin A1, with negative CD3, CD5, CD23, CD10, cyclin D1 and *BCL6*.

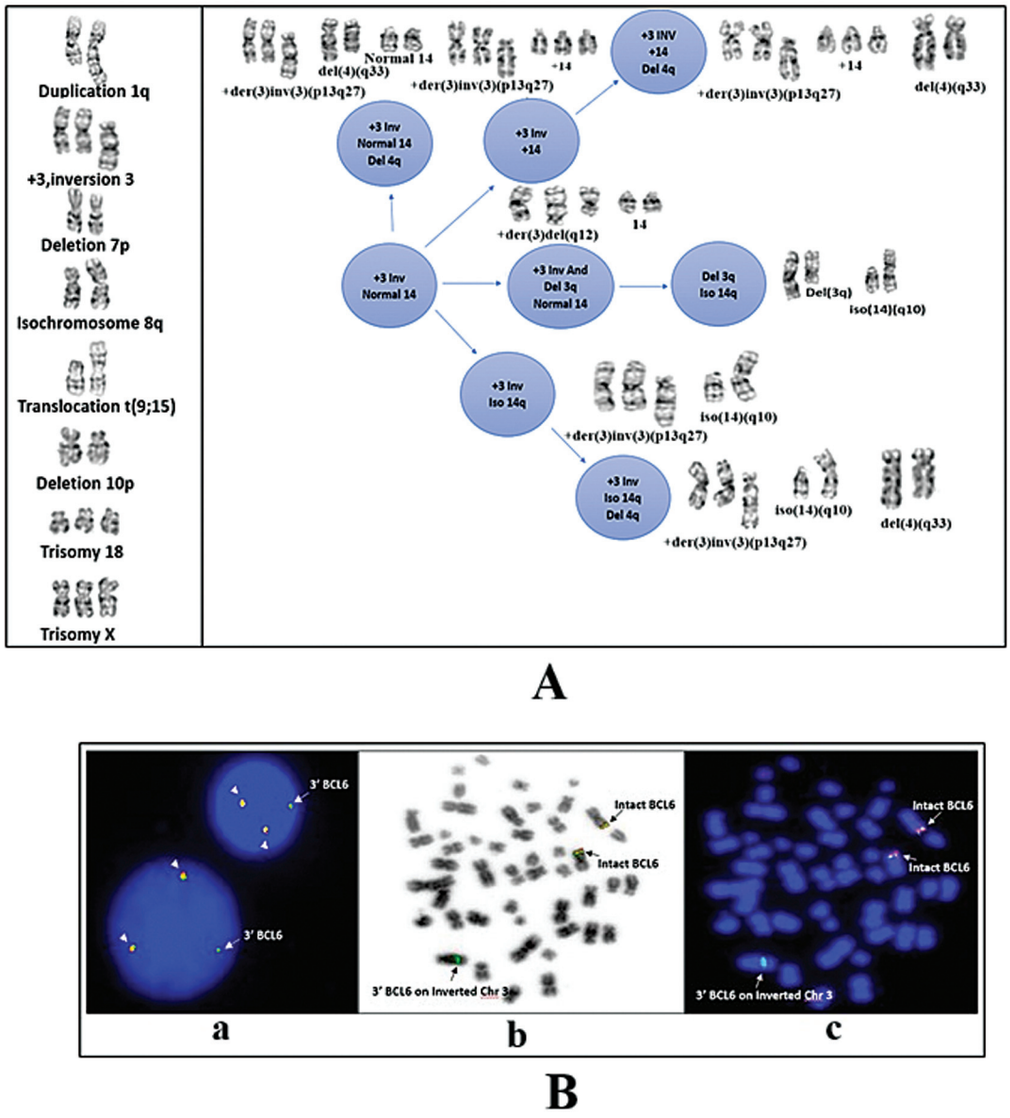

Fig. 2 (A) Representative karyotype image showing a complex karyotype with multiple clonal structural and numerical abnormalities. (**B**) FISH on interphase (a) and metaphase cells (b; inverted DAPI, d; DAPI) revealed a *BCL6* rearrangement, with intact yellow fusion signals on two normal chromosome 3 and an inverted chromosome 3 showing loss of the spectrum orange (5′) signal and retention of the spectrum green (3′) signal, consistent with an inversion of *BCL6* gene.

An atypical variant *BCL6* gene rearrangement was detected with three copies of the *BCL6* gene, on two normal chromosome 3 and one on derivative 3 as a result of 3q27 inversion, resulting in *BCL6* rearrangement with loss of the 5′ Spectrum orange signal (►**Fig. 2B**).

Molecular testing was negative for the BRAF mutation.

Based on the diagnosis, the patient was initiated on Rituximab and Bendamustine, planned for 6 monthly cycles. However, after the first cycle, she developed sepsis with disseminated intravascular coagulation, bilateral pleural effusion, persistent fever, and altered consciousness. CT brain showed progression of hemorrhages with new hemorrhagic foci due to severe thrombocytopenia at presentation and sepsis following chemotherapy. Advanced imaging could not be performed due to the patient's critical postchemotherapy condition and requirement for mechanical ventilation. Despite rituximab–bendamustine therapy and intensive supportive care, including ICU admission, oxygen support, transfusions, and antibiotics, the patient developed refractory cytopenias, recurrent sepsis, progression of intracranial bleed, and seizures requiring mechanical ventilation. Due to a complicated clinical course, she ultimately succumbed to sepsis with multiorgan failure, reflecting the aggressive nature of the disease that posed challenges in management.

Discussion

The diagnosis of SBLPN, newly classified as HCL-V, remains challenging due to overlapping features with other B-cell neoplasms.[5]

Considering the differential diagnosis of splenic marginal zone lymphoma versus SBLPN, the presence of large prolymphocytes, absence of BRAF V600E mutation, and lack of CD5 expression supported the diagnosis of SBLPN.

We identified a novel double-hit mechanism involving the *BCL6* gene, with the presence of two distinct divergent *BCL6*-abnormal clones within a complex karyotype. One clone harbored an extra copy of derivative chromosome 3 with inv(3)(p13q27), leading to an atypical *BCL6* rearrangement with 5′ deletion, while the other showed a 3q deletion encompassing the *BCL6* locus with a separate pathogenic tumor suppressor mechanism. The karyotype revealed clonal evolution from a complex stemline clone of extra copy of chromosome 3 with inversion, 1q duplication, isochromosome 8q, deletions of 7p and 10p, a derivative chromosome 9 resulting from t(9;15) and 9p deletion, trisomy 18, and trisomy X. As illustrated in the figure (►**Fig. 2A**), subclonal trajectories evolved from this stemline to acquire additional aberrations such as 3q deletion, trisomy 14, isochromosome 14q, trisomy 14 and 4q deletion reflecting genetic instability.

The copy number alterations in this case, that is, gain of 1q, trisomy 3, trisomy 18, partial trisomy 8q, and deletion of 7p that have been independently linked to disease progression and poor prognosis in aggressive lymphomas, such as diffuse large B cell lymphoma (DLBCL).[2]

Although complex karyotypes are frequently observed in SBLPN, the cytogenetic profile in the present case deviated from the classical alterations reported in HCLv, that is, abnormalities involving 5q, 14q32, 8q24, deletions of 17p and 7q, and trisomy 12.

We observed partial trisomies of 1q, and 8q and complete trisomies of chromosomes 3, 14, 18, and X, along with losses in 9p and 10p.

Chromosome 1 abnormalities are common in human cancers, with 1q gain (+1q) being the most frequent secondary aberration in Burkitt lymphoma, often associated with aggressive disease.

Notably, 1q gain has not been reported in classical or variant HCL, making its duplication in this case a novel finding with potential prognostic significance.[6]

Additionally, the presence of two distinct clones involving chromosome 14, one with trisomy 14 and the other with isochromosome 14q;i(14q), suggests parallel clonal evolution involving the same chromosomal region through different mechanisms, both resulting in increased gene dosage.

While 3q27 translocations are common in B-cell neoplasms, the present case represents the first reported SBLPN with a novel inversion, inv(3)(p13q27).

A similar inversion involving 3p24 and 3q27 has been described in only two cases according to the Mitelman database; one in follicular lymphoma and another in undifferentiated pleomorphic sarcoma. Thus, our finding expands the spectrum of 3q27 rearrangements in SLBPN and introduces 3p13 as a novel partner region.[7,8]

BCL6 (3q27) is an oncogene encoding a transcriptional repressor critical for germinal center B cell development and immune responses. Rearrangements occur in 19 to 30% of DLBCL, which deregulate *BCL6* and drive lymphomagenesis.

The involvement of 3p13 in our case expands the spectrum of *BCL6* partner loci, highlighting inversion 3 as a potential atypical mechanism of *BCL6* dysregulation. To date, over twenty *BCL6* translocation partners have been identified, typically involving immunoglobulin gene enhancers. Further investigation to define the unrecognized partner gene at 3p13 is warranted to elucidate its pathogenic role. Although this finding may have prognostic and therapeutic significance similar to *BCL6*'s role in DLBCL, long-term follow-up is essential to inform clinical management.

Meta-analyses link *BCL6* rearrangements to poor prognosis in DLBCL, particularly with rituximab-based therapy, underscoring the importance of reporting such findings in SBLPN for prognostic significance.[9,10]

Subclonal *BCL6* rearrangement with partial 5′ deletion is a novel disruption mechanism affecting *BCL6* expression and pathogenesis.

BCL6 translocations in follicular lymphoma and DLBCL involve the major translocation cluster at the 5′ end, a hotspot for somatic hypermutation, causing balanced promoter substitutions, deregulating expression, and promoting lymphomagenesis by blocking differentiation and apoptosis. In the present case, the telomeric (5′) region of *BCL6* was lost (absent orange FISH signal), while the centromeric (3′) region remained intact and rearranged. The intrachromosomal inversion, inv(3)(p13q27), represents an unusual unbalanced *BCL6* rearrangement that repositions the 5′ promoter and exon 1 under the regulatory influence of a novel partner at 3p13, likely causing promoter substitution and transcriptional deregulation. Despite loss of the classical 5′ coding region, the retained 3′ segment may allow aberrant or chimeric expression, contributing to lymphomagenesis.

Another notable finding was the absence of *BCL6* protein expression on immunohistochemistry (IHC). Such discordance, reported in rare DLBCL cases, may result from epigenetic silencing, nonfunctional fusion transcripts, or technical limitations of IHC.[11]

SBLPN typically follows an aggressive clinical course and shows resistance to Cladribine monotherapy, though improved responses have been reported with combination regimens such as Rituximab or Bendamustine.[12,13]

Hence, this clinically aggressive and genetically complex case necessitated a comprehensive evaluation to clarify diagnosis, define prognosis, and guide treatment.

Conclusion

Given the ongoing debate surrounding the classification of SBLPN and its overlap with entities such as HCL-V and B cell neoplasms, an integrated diagnostic approach, encompassing clinical, morphological, histological, immunophenotypic, and genetic data, is essential for accurate diagnosis and effective management. Early multidisciplinary involvement and considering individualized treatment plans with potential modifications in chemotherapy could possibly improve outcomes, but salvage is often difficult in such advanced cases.

Incorporating such insights into diagnostic frameworks will be pivotal in advancing these entities from provisional

status to well-defined categories in current guidelines with evidence-based, targeted treatment options.

Authors' Contributions

S.M.A.A.: Concept, design, experimental studies, data analysis, manuscript preparation, manuscript editing, and manuscript review.
A.P.: Concept, literature search, data analysis, manuscript preparation, manuscript editing, and manuscript review.
R.H.M.I.: Experimental studies, hematopathology study, data analysis, manuscript editing, and manuscript review.
U.Z. and N.M.B.: Clinical study, treatment, data analysis, manuscript editing, and manuscript review.

Conflict of Interest

None declared.

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Richter's Transformation in an Unusual Site: Non-Hodgkin Lymphoma in the Parotid Region

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To the editor,

A 73-year-old female patient, a known diabetic, presented to our tertiary care center with swelling on the right side of the face for the past 1 month, insidious in onset and gradually increasing in size. The patient had a past history of chronic lymphocytic leukemia (CLL) that was diagnosed at an outside hospital 3 years ago, for which she was taking treatment comprising of tablet chlorambucil. Further details of initial diagnosis were not known. Her Eastern Cooperative Oncology Group performance status indicated a score of 0 (able to perform all daily activities). Upon hematological investigations, she had a hemoglobin of 13.6 g%, total count of 56,800 cells/cu.mm, platelet counts was 3.29 lakhs, and serum lactate dehydrogenase level was 163 U/L (normal: 208–370 U/L). Uric acid and aspartate aminotransferase levels were low. Bone marrow biopsy was not performed. Computed tomography and positron emission tomography showed a fluorodeoxyglucose (FDG)-avid mass lesion with mandible erosion in the right parotid with a maximum standardized uptake value of 11.8 (►**Fig. 1A, B**). The scan also showed

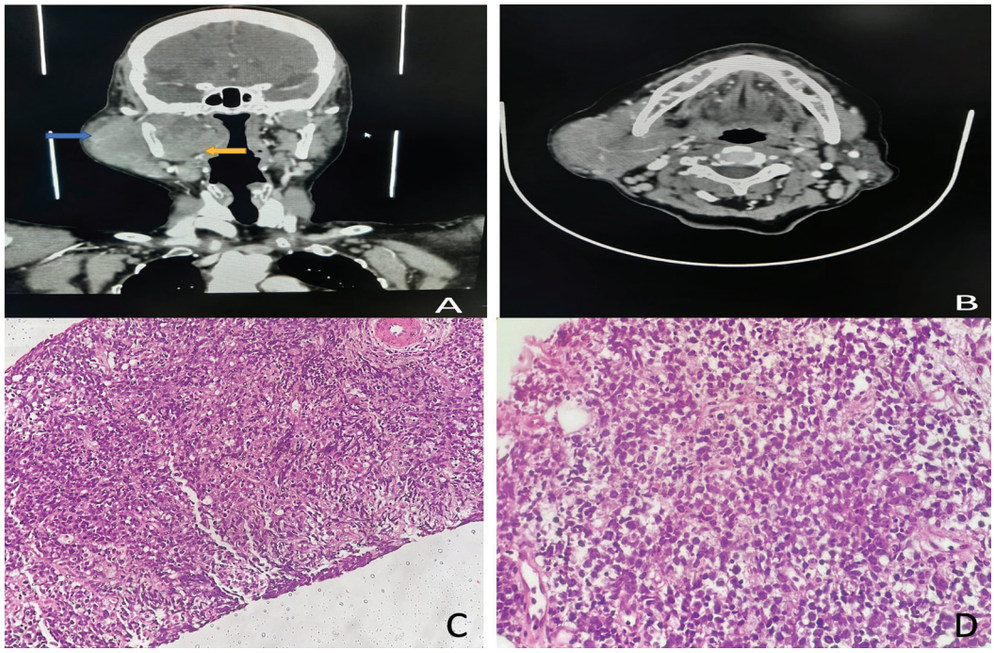

Fig. 1 (**A** and **B**) Computed tomography of the neck with contrast showing an ill-defined homogeneously enhancing soft tissue density involving the superficial lobe (blue arrow) and deep lobe (yellow arrow) of the right parotid gland with extensions. (**C** and **D**) Hematoxylin and eosin-stained sections in 20× (**C**) and 40× (**D**) magnifications, respectively, showing infiltration by sheets of atypical large lymphoid cells, with the presence of brisk mitosis and apoptosis.

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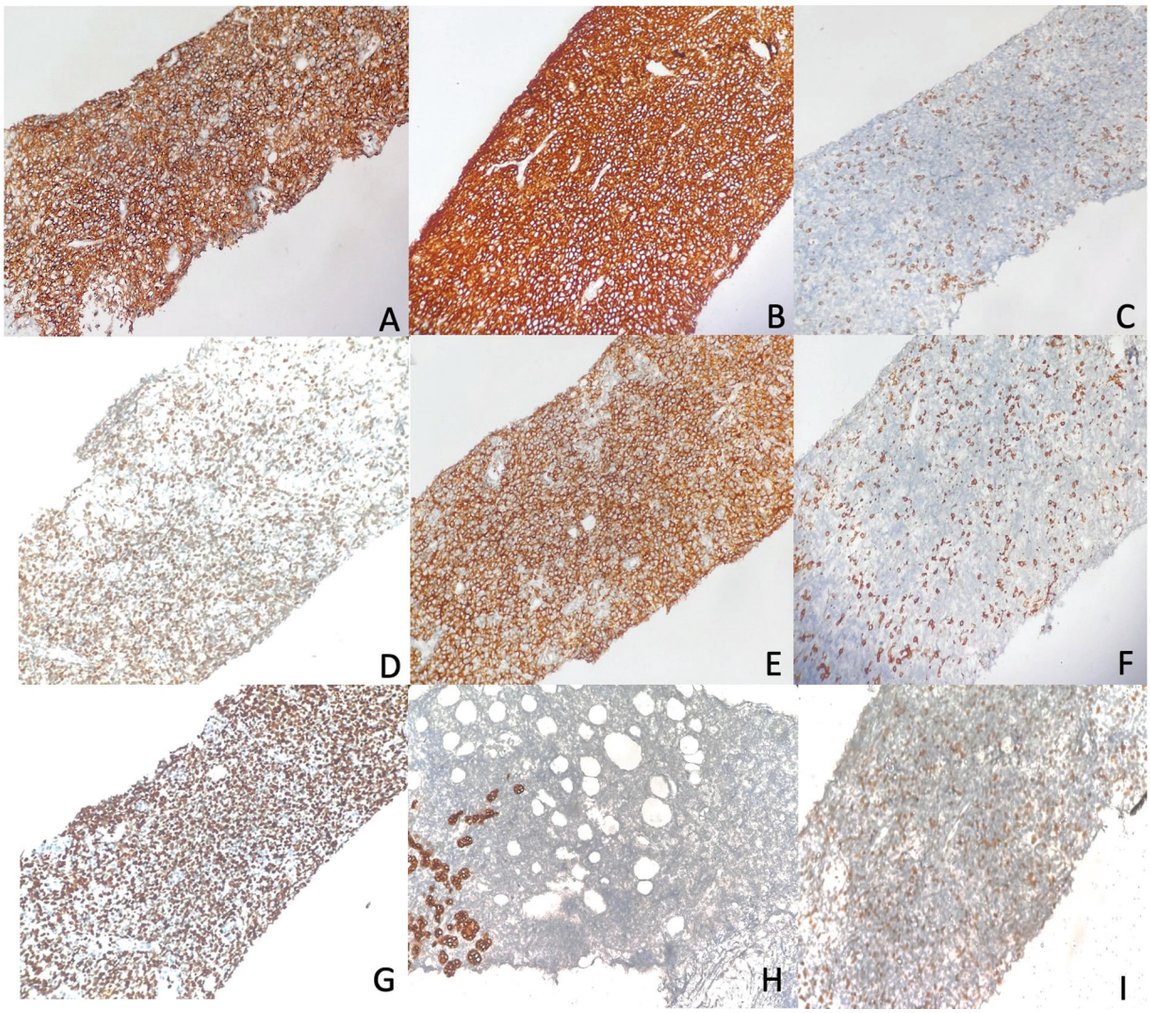

Fig. 2 Immunohistochemical findings in the tumor cells in 20 ×: (**A**) CD45 positive in the tumor, (**B**) CD20 diffusely positive in the tumor cells, (**C**) CD3 scattered positivity noted, (**D**) Bcl6 positive, (**E**) Bcl2 diffusely positive, (**F**) CD5 positive in scattered cells, (**G**) Ki67 80% in the tumor cells, (**H**) cytokeratin positive in entrapped glands and negative in tumor cells, and (**I**) C-myc focally positive.

multiple mildly prominent and mildly enlarged lymph nodes in the neck and bilateral axilla, with a maximum size of 15 mm on the right and 17 mm on the left and pelvis with no minimal FDG uptake. An image-guided accurate biopsy from the right parotid gland was performed.

Grossly, we received six linear cores of soft tissue, the largest measuring 0.5 cm and the smallest measuring 0.2 cm. Upon microscopic examination, sections showed diffuse infiltrating lesion composed of sheets of large cells with scant cytoplasm, increased nucleocytoplasmic ratio, nuclear pleomorphism, and coarse chromatin. Brisk mitotic figures and apoptotic bodies were also seen (►**Fig. 1C, D**).

Immunohistochemistry (IHC) performed showed diffuse positivity for CD45, CD20, BCL6, and BCL2 and focally for CD5 in the large cells, while they were negative for CD3, CD10, CK7, cyclin D, and MUM1. Ki67 labeling index was 80% (►**Fig. 2A–I**). The IHC findings confirmed the diagnosis of a diffuse large B cell lymphoma (DLBCL) germinal center type, involving the parotid gland, establishing a Richter's transformation from CLL. Following this, she was again started on rituximab-containing chemotherapy that also included injection doxorubicin, vincristine, and cyclophosphamide. However, she finally succumbed to the disease 3 months later.

Richter's transformation refers to a phenomenon in which CLL or small lymphocytic lymphoma transforms into a more aggressive form of lymphoma, usually DLBCL, with incidence rates of 2 to 10%.[1] The exact cause has yet to be understood, but mutations such as *TP53*, *c-Myc*, and *NOTCH1* have been documented.[2,3] Richter transformation occurs in salivary glands such as the submandibular gland; however, it is extremely rare to occur in the parotid gland.[4] The prognosis for such cases is generally less favorable, and treatment is challenging. Hence, a high index of suspicion is crucial for a timely diagnosis and to plan appropriate treatment. Recent therapies such as chimeric antigen receptor T-cell therapy and treatment with monoclonal antibodies have demonstrated encouraging results and are in the clinical trial stage.[5] This case was presented due to its rare location and distinct clinicopathological implication.

Authors' Contributions

J.J. was involved in literature search, data acquisition, data analysis, manuscript preparation. A.B. was involved with the concept, design, definition of intellectual content, clinical study, manuscript editing, review and guarantor. L.D. and N.R. were involved with definition of intellectual content, clinical study, manuscript review and editing.

Patient Consent

Patient consent is not required due to the retrospective nature of the study.

Conflict of Interest

None declared.

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Role of Aflatoxins in Cancer of Gallbladder in North India

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Dear Sir,

I read with interest the hypothesis by Boby and Mathew about the possibility of microplastics being involved in the gallbladder cancer (CAGB) hotspot along the Gangetic basin.[1] Microplastics are ubiquitous, not limited to the Ganges, and as the authors admit, there is little or no evidence to link them with cancer of any system. As an oncologist who worked in North India, I found that very few patients drink water directly from the Ganges; their water supply, depending on the area of residence, is either piped supply or wells. It is also pertinent to note that this CAGB hotspot includes regions where the Ganges does not flow, ranging from northeast India, Pakistan,[2] and Nepal.[3]

The strongest link to CAGB is cholesterol gallstones (overlooked by the authors) and there is a definite dichotomy here, with North Indians (especially women) being more prone to cholesterol gallstones and South Indians to pigmentary stones; this could be due to genetic (such as ATP binding cassette transporter B1 [*ABCB4*] mutations mentioned by the authors) or dietary factors. Excess cholesterol in the human body is treated as a toxin and pumped out into bile by the ATP-binding cassette transporter system, the same system is used for other xenobiotics.[4] In 2008, I had hypothesized that a hepatocarcinogen like aflatoxin B (common across India due to poor food storage practices) could be pumped out along with cholesterol in individuals prone to cholesterol gallstones and thus explain the association between cholesterol gallstones and CAGB.[5] Aflatoxin contamination is common in food items such as rice, wheat, peanuts, maize, and even red chili peppers in India, where storage in hot humid conditions is conducive to the growth of the fungus, Aspergillus spp.[6]

Since then, there have been epidemiological studies from hotspots such as Chile[7], China,[8] and India[9] confirming this association. The trend in incidence of this cancer in future will provide a clue, as a fall would indicate the role of a preventable food contaminant (as food hygiene practices have been improving over the last few decades), and a rise, possibly microplastics. CAGB is a serious concern across North India as it disproportionately affects women of reproductive age and shatters family dynamics, and more research is urgently required to pinpoint the cause.

Conflict of Interest
None declared.

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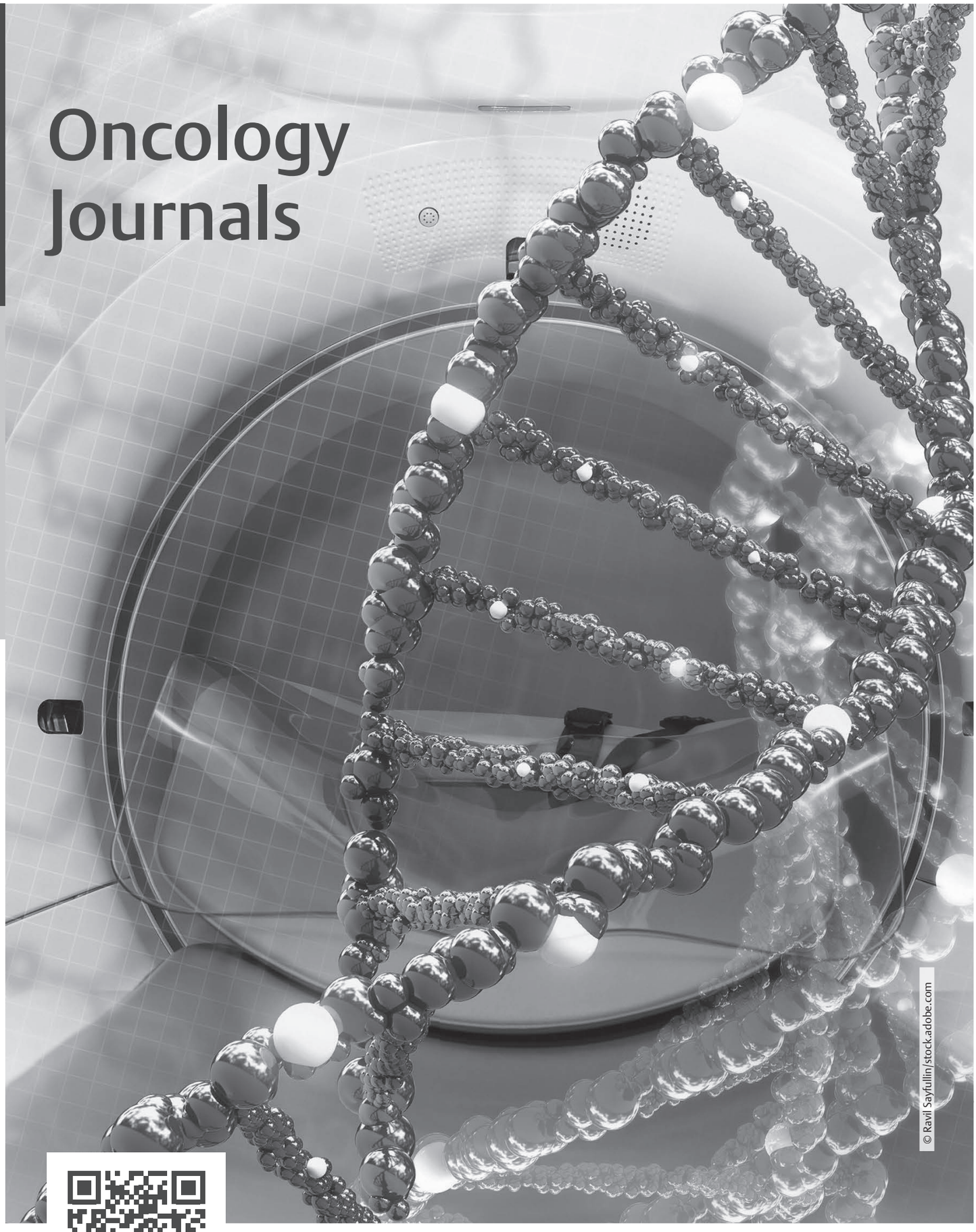

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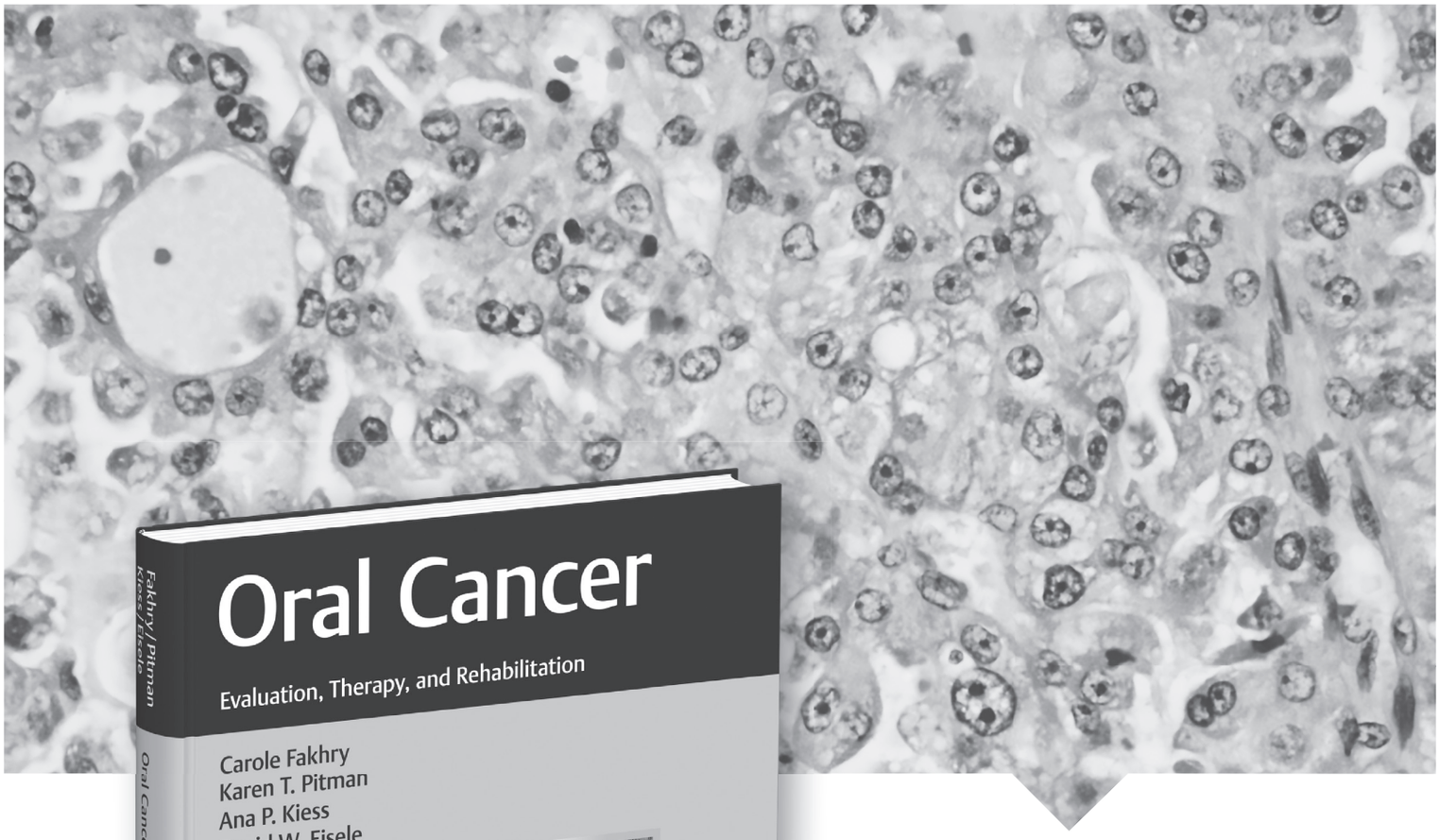

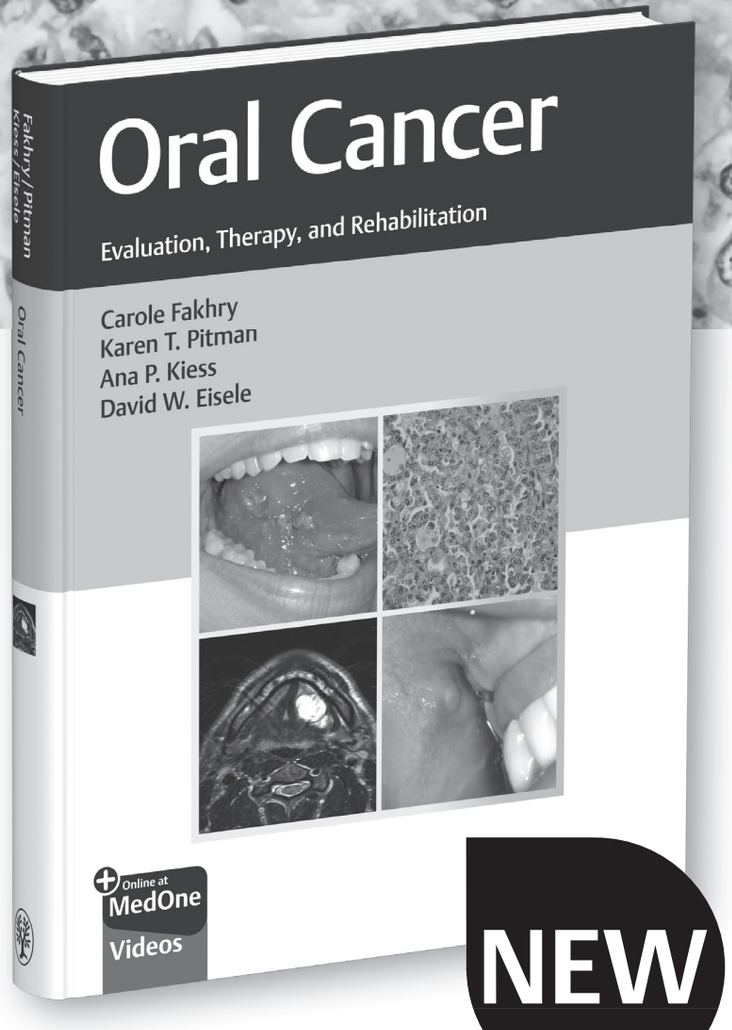

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