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Advances in Cancer Diagnosis and Treatment

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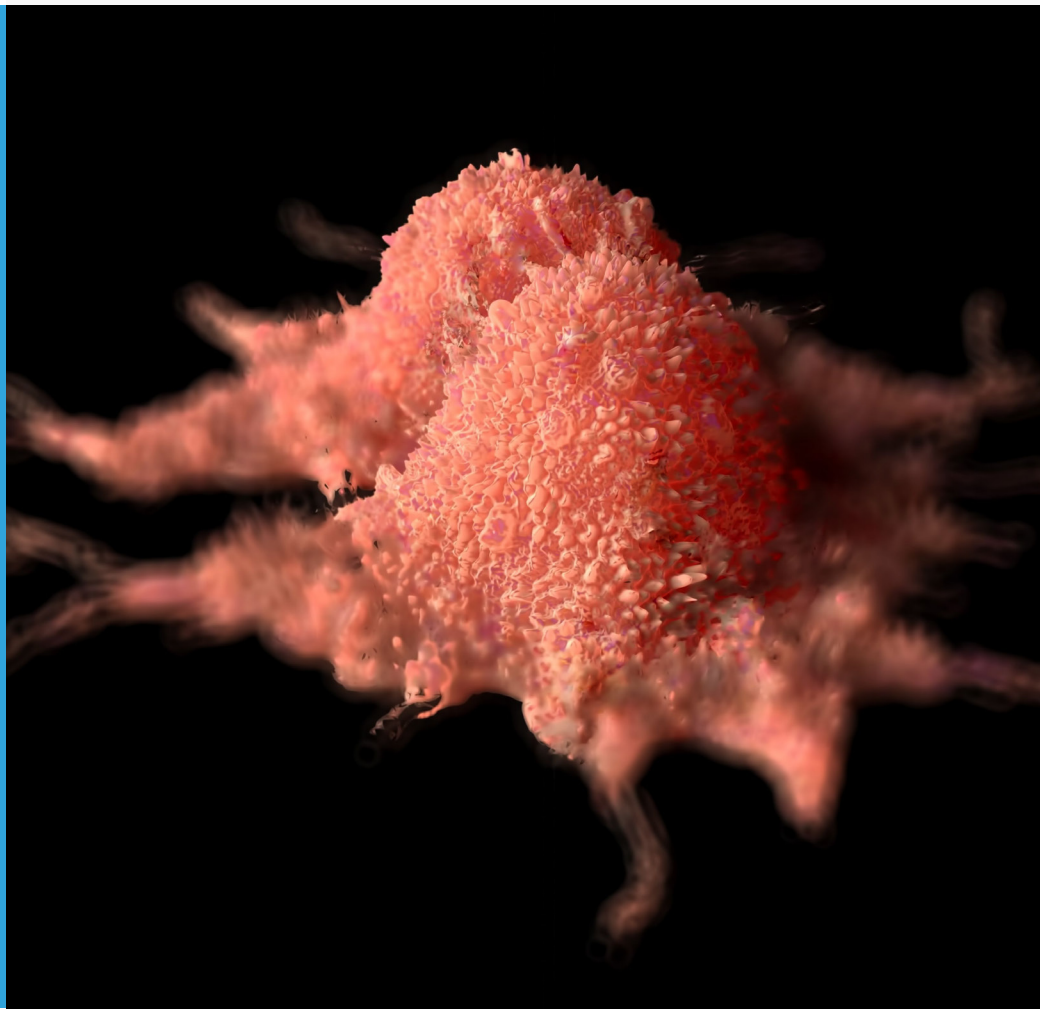

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Advances in Cancer Diagnosis and Treatment

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More people are surviving cancer than ever before, in part due to advances in diagnosis and treatment. Since the COVID-19 coronavirus pandemic, an unprecedented strain has been placed on healthcare providers to manage the backlog and demand for cancer services. In response, the NHS has published an ambitious long term plan to diagnose cancer at an earlier stage and improve the five year survival rate, maintaining the decade-on-decade fall in mortality rates previously seen.

This special issue of the British Journal of Biomedical Science highlights the latest research and development in the diverse field of cancer, and how these innovations move our understanding forwards, leading to improvements in the diagnosis, monitoring and treatment of these conditions.

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SPECIAL ISSUE EDITORIAL
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Editorial: Advances in Cancer Diagnosis and Treatment

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Keywords: cancer biomarkers, cancer diagnosis, cancer treatment, novel diagnostics, novel biomarkers, oncology, cancer test

Editorial on the Special Issue

Advances in Cancer Diagnosis and Treatment

This Special Issue of the *British Journal of Biomedical Science* brings together a collection of articles which all contribute to the advancement of knowledge into the diagnosis and treatment of cancer. Each article in this diverse research field offers an insight into current clinical practice or provides evidence for a potential future novel diagnostic test or treatment. Each manuscript presented here has the potential to contribute directly towards improving patient outcomes.

Cancer is a burden on society across the world. Cole et al. presented work focusing on colorectal cancer within the United Kingdom, while Liau et al. conducted work focusing on colorectal cancer in Malaysia. Both groups of authors highlighted that colorectal cancer in their studied populations has a high prevalence and mortality rate.

Cole et al. reviewed the clinical utility of one of the current tests used in the screening and diagnosis of colorectal cancer. The faecal immunochemical test (FIT) detects haemoglobin in a person's faeces and is used to determine if occult bleeding is present in the gastrointestinal tract and whether further invasive follow-on testing is required. Studying a large population in the north of England, Cole et al. presented evidence showing that 8% of FIT tests in people later confirmed to have colorectal cancer were incorrectly classified as negative. The authors point out that this only accounts for 0.06% of all FIT performed in this population, and that the test still has a very high diagnostic sensitivity, but highlight the need for improving diagnostic tests.

The review by Liau et al. on colon cancer-associated transcript-1 (CCAT-1) goes some way towards providing pleasing evidence for a new diagnostic test for colorectal cancer. This long non-coding RNA (lncRNA) was originally found to be over-expressed in colorectal cancer, but has now been shown to be over-expressed in many types of cancers. Liau et al. are keen to stress that there is a considerable amount still not known about CCAT-1, including its mechanism of action and factors that cause its dysregulation, but highlight that its downregulation may be correlated with drug sensitivity and better treatment outcomes.

Continuing the theme of manipulating a patient's RNA to improve chemotherapy drug sensitivity, Wodi et al. demonstrated the potential of a novel therapeutic in the Kasumi-1 cell line model of acute myeloblastic leukaemia. Wodi et al. used a 3-(trifluoromethyl)anilide scaffold named SPHINX to inhibit the activity of the splice factor protein kinase in a cell culture model of leukaemia, thereby manipulating alternative splicing. This splice factor protein kinase regulates the activity of SRSF1, an important SR protein splice factor regulator of alternative splicing of many critical cancer-associated genes. Alternative splicing, in which one multi-exon gene can produce many different mRNA transcripts, via skipping or retaining introns and exons, is a widespread process that accounts for some of the complexity of the proteome. Dysregulated alternative splicing is

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a feature of many cancers, and the authors present compelling evidence that splice factor kinase inhibition could bring therapeutic benefits.

Remaining on the theme of cancers of the blood and bone marrow, Al-Zubaidi and Hughes investigated the role of a new biomarker using immunophenotyping flow cytometry to help differentiate between different B-cell lymphoproliferative disorders. The authors begin with a succinct overview of the World Health Organisation classification of B-cell lymphoproliferative disorders and summarised the difficulties in distinguishing between these heterogeneous leukaemias and lymphomas. Highlighting how an atypical presentation of chronic lymphocytic leukaemia can cause diagnostic uncertainty, the authors presented further evidence for the role of CD200 in diagnostic algorithms. The authors outline the case to give further support for including CD200 in routine immunophenotyping testing panels.

Focusing on white blood cells of the myeloid lineage, Chohan et al. presented a meta-analysis of oral squamous cell carcinoma (OSCC) and biomarkers that may be useful in determining prognosis. Focusing on tumour associated macrophages and the role they play in OSCC, the authors investigated the role of CD68 and CD163 expression by these cell types along with PD-L1. The authors present evidence suggesting that CD163 positive tumour associated macrophages were connected with a poor prognosis in OSCC, but CD68 positive macrophages had no correlation with prognosis. The authors also found that raised PD-L1 may have a positive impact on prognosis, but warn that whether this expression is located in the tumour or stromal cells may be a significant factor. However, the authors conclude that the available evidence is currently too weak to support claims on the utility of PD-L1. The authors completed their analysis by contrasting their findings in OSCC to the results seen in studies of other cancers.

Murugan and Alzahrani analysed almost 15,000 solid malignancy samples across 37 cancer types stored in the cancer genome atlas, looking for mutations in the genes for isocitrate dehydrogenases 1 and 2. The authors found that approximately 3% of cancers overall contained mutations in isocitrate dehydrogenases 1 (*IDH1*), but this rose to 34% in gliomas. Linking these mutations to prognosis, the authors presented evidence to show that patients with a mutation in *IDH1* had an improved overall survival rate and better progression free survival. The authors conclude their paper by linking their work on mutant *IDH1* and 2 with the effectiveness of the inhibitors ivosidenib and enasidenib, used in the treatment of acute myeloid leukaemia. They hypothesise that there may also be a key therapeutic potential in targeting *IDH1* and 2 in gliomas and other malignancies.

These six papers, although different in subject areas, aims and methodologies, each contribute to the ongoing development of new diagnostics and treatments for cancer.

AUTHOR CONTRIBUTIONS

This editorial was written by lead guest editor MH. It was then reviewed and edited by ML, QW, and NP. All authors contributed to the article and approved the submitted version.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

BIOMEDICAL SCIENCE IN BRIEF
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Faecal Immunochemical Test (FIT) Sensitivity; A Five Year Audit

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Introduction: Colorectal cancer has a high prevalence and mortality rate in the United Kingdom. Cancerous colorectal lesions often bleed into the gastrointestinal lumen. The faecal immunochemical test (FIT) detects haemoglobin (Hb) in the faeces of patients and is used as a first line test in the diagnosis of colorectal cancer.

Materials and Methods: A retrospective audit of all FIT performed and all colorectal cancers diagnosed in the Hull and East Riding of Yorkshire counties of the United Kingdom (population approximately 609,300) between 2018 and 2022 was conducted. FIT were performed using a HM-JACKarc analyser from Kyowa medical. The predominant symptom suggestive of colorectal cancer which prompted the FIT was recorded. Colorectal cancer was diagnosed using the gold standard of histological biopsy following colonoscopy.

Results: Between 2018 and 2022, 56,202 FIT were performed on symptomatic patients. Follow on testing identified 1,511 with colorectal cancer. Of these people, only 450 people with a confirmed colorectal cancer had a FIT within the 12 months preceding their diagnosis. Of these 450 FIT results, 36 had a concentration of <10 µg/g and may be considered to be a false negative. The sensitivity of FIT in the patients identified was 92.00%. The most common reason stated by the clinician for a FIT being performed in patients with colorectal cancer was a change in bowel habits, followed by iron deficient anaemia. The number of patients diagnosed with colorectal cancer decreased in 2020, but increased significantly in 2021.

Discussion: This study shows that 8.00% of people diagnosed with colorectal cancer in the Hull and East Riding of Yorkshire regions had a negative FIT. This study also shows that the SARS-CoV-2 pandemic affected the number of people diagnosed with colorectal cancer, and therefore skews the prevalence and pre-test probability of a positive test. There are many reasons why a FIT could produce a false negative result, the most likely being biological factors affecting the stability of haemoglobin within the gastrointestinal tract, or pre-analytical factors influencing faecal sampling preventing the detection of haemoglobin. Some colorectal lesions do not protrude into the gastrointestinal lumen and are less likely to bleed.

Conclusion: This is the first study showing data from outside of a structured clinical trial and provides the largest study to date showing the sensitivity of FIT in a routine clinical setting. This study also provides evidence for the impact COVID-19 had on the rate of colorectal cancer diagnosis.

Keywords: colorectal cancer, FIT, faecal immunochemical test, sensitivity, bowel cancer, COVID-19, SARS-CoV-2, intestine

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INTRODUCTION

Colorectal cancer is the fourth most common cancer in the United Kingdom [1]. This accounts for more than 40,000 new cases and almost 17,000 deaths [1]. Colorectal cancer also poses a significant financial burden to the United Kingdom, in 2020 it was reported that colorectal cancer cost the UK economy £1.7 billion a year [2]. This cost is a combination of the direct cost from the sum of all healthcare provided and indirect cost from people of working age who are on unable to work, forced into early retirement, or do not survive.

Blood in the stool is a common symptom of colorectal cancer [3]. The Faecal Immunochemical Test (FIT) is a laboratory investigation used to detect haemoglobin (Hb) in faeces, even when the blood may not be visible [3]. The National Institute for Health and Care Excellence (NICE) published its DG30 guidelines for "quantitative faecal immunochemical tests to guide referral for colorectal cancer in primary care" in 2017 [4]. These guidelines state that FIT should be used in patients with a low pre-test probability, but symptomatic of colorectal cancer. It advises the use of FIT for triaging patients before a colonoscopy is performed and that patients with a positive FIT result should be given urgent priority. In 2023, following a publication by D'Souza et al [5], NICE produced the DG56 guidelines recommending that patients with both high risk and low risk symptoms, and therefore both a high and low pre-test probability for cancer should have a FIT test performed.

A number of large clinical trials have reported that FIT has a very high diagnostic sensitivity. In some of these trials, FIT has been reported to have a sensitivity of: 97% (D'Souza et al [5]), 100% (Godber et al [6]), >99% (Ng et al [7]), 100% (Westwood et al [8]), and 100% (McDonald et al [9]). Therefore, a negative result is believed to provide assurance that a person does not have a lower gastrointestinal cancer.

This audit reviewed the diagnostic sensitivity of FIT in all patients diagnosed with colorectal cancer in Hull and East Yorkshire over a 5 year period.

MATERIALS AND METHODS

We performed a search of all patient records stored on the Hull University Teaching Hospitals laboratory information management system (LIMS), (Labcentre, Clinisys, Tucson, United States). We identified all patients who had colorectal cancer diagnosed between 2018 and 2022. We searched for patients using the SNOMED codes shown in **Appendix 1**.

The current gold standard for the diagnosis of colorectal cancer was used. All patients with suspected colorectal cancer had a colonoscopy performed and a biopsy taken. Histological examination of the biopsy sample confirmed the diagnosis of cancer.

Colonoscopy and biopsies were performed by Hull University Teaching Hospitals NHS Trust. Macroscopic and microscopic examination of all biopsy samples was performed. Biopsy specimens taken from the gastrointestinal tract were fixed in neutral buffered formalin for 24 h, resections were fixed for 2–3 days. After dissection, specimens were dehydrated using alcohol, then xylene, and finally embedded in paraffin wax, all using an automated Leica Peloris rapid tissue processor (Milton Keynes, United Kingdom). A Leica Rotary microtome was used to section the embedded tissue samples and the sections were then stained by a Dako (California, United States) automated haematoxylin and eosin stainer. All specimens were analysed by an NHS Consultant Histopathologist as part of routine care.

FIT results, when available, were obtained from the LIMS for all patients with a confirmed diagnosis of colorectal cancer. FIT performed up to 1 year prior to the biopsy were included. FIT performed after the biopsy or greater than 1 year before the biopsy were excluded.

FIT testing was performed within the UKAS ISO 15189 accredited Pathology Laboratory at Hull University Teaching Hospitals using a HM-JACKarc analyser (Kyowa medical, Japan). Calibration and quality control materials were provided by Alpha labs (Hampshire, United Kingdom). Patients collected their own specimen into faecal collection devices (Alpha labs, United Kingdom) containing stabilising buffers to prevent sample degradation [10]. Polyclonal antibodies specific to the globin fraction of Hb bind to any Hb present in the specimen resulting in a turbidimetric change proportional to the concentration. A cut-off value of 10 µg/g of haemoglobin in faeces was regarded as a positive result.

Along with all FIT, the clinician recorded the primary presenting symptom which prompted the suspicion of colorectal cancer. One of five symptoms, all linked to the NICE DG30 guidelines were recorded: unexplained abdominal pain [I], unexplained weight loss [II], changes in bowel habit [III], iron deficient anaemia [IV], or anaemia in the absence of iron deficiency [V].

This is an audit of patient outcomes during routine clinical care, all results generated were part of the patient's standard treatment. This study was a clinical audit approved by Hull University Teaching Hospitals NHS Trust, reference number BIOC/SE/2024-25/01.

RESULTS

FIT Testing and Patient Demographics

The Pathology Laboratory at Hull University Teaching Hospitals performed 56,202 FIT tests in the 5 year period between 2018 and 2022. Of these tests, 41,914 results were <10ug/g and therefore negative and 2009 results were >400 ug/g; this is positive and above the measurement range of the instrument. The remaining specimens which produced a reportable result, the mean result was 67 ug/g. During this time period 1,511 patients were diagnosed with colorectal cancer by colonoscopy and histology. Demographics of the people diagnosed with cancer where as follows; 896 male (59.3%), 615 female (40.7%). The age range of the patients was from 24 to 97, median age 70 years old (IQR 62–77). See **Figure 1**.

FIT Sensitivity

Of the 1,511 patients diagnosed with colorectal cancer, only 450 had a FIT performed. Of these patients, it was found that 36 patients with colorectal cancer had a false negative FIT result, giving a sensitivity of 92.00%. **Figure 2** shows a breakdown of the false negative FIT rate by year. It can be seen that there is a post COVID-19 pandemic rise in the number of patient's diagnosed with colorectal cancer.

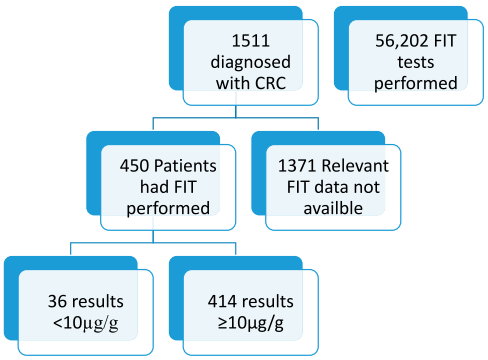


FIGURE 1 | Figure shows total number of patients included and excluded in this study and the availability of FIT data.

The 36 patients identified includes 22 male (61%) and 14 female (39%). The median and mean ages were 72 and 71.9 years respectively. Blood haemoglobin results pre-admission where reviewed where possible. The median male and female blood haemoglobin results were 127 g/L and 120 g/L, respectively.

Of these 36 patients with FIT negative colorectal cancer, 6 had a histological report that described the removed mass as sessile, that is flat and not protruding into the lumen of the gastrointestinal tract.

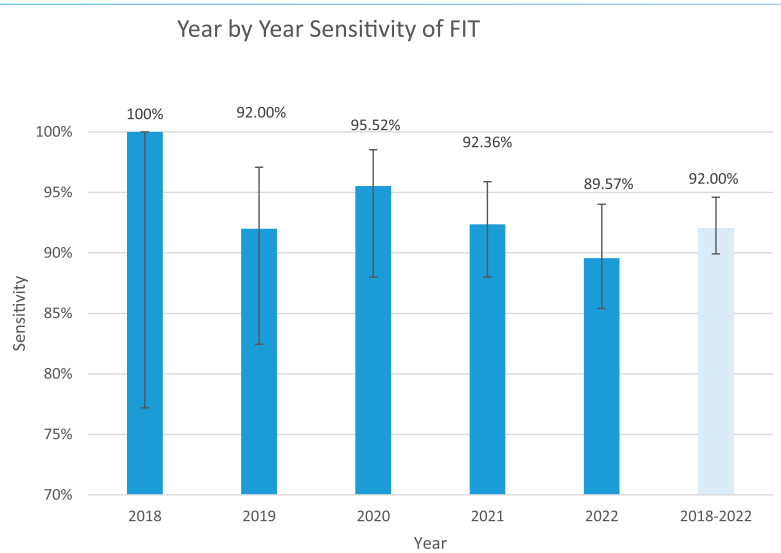
Presenting Symptoms

Table 1 shows the most common reasons why a patient's NHS General Practitioner requested a FIT. Data was reported for 387 patients who were later diagnosed with colorectal cancer. It can be seen that approximately half of all patients (51.4%) with confirmed colorectal cancer were reported to have a change in bowel habit, and one-fifth (22.5%) were reported to have iron deficient anaemia as the symptom that prompted a FIT.

DISCUSSION

This manuscript is the first report on the sensitivity of FIT in a routine clinical setting. Our audit of FIT used under real world conditions, outside of a trial, shows that false negative results are rare but do occur. Our finding that the sensitivity, when using a cut-off value of 10 µg/g, is 92.00%. This finding is in agreement with the reported findings of the clinical trials of Mowat et al [11] 86.7%, Chapman et al [12] 87.5%, Vieto et al [13] 90.8% and Shaukat et al [14] 91%. Using a cut off of <15 µg/g, Katsoula et al [15] reported a sensitivity of 93%, again this is similar to our findings.

Our finding show that a negative FIT result cannot rule out colorectal cancer. It is now commonly adopted clinical practice for a negative FIT result to be used to triage and downgrade a patient's need for an urgent colonoscopy [16]. The Association for Coloproctology of Great Britain and Ireland suggest that FIT



Year	Patients Diagnosed with CRC	FIT Tests	False Negatives (<10µg/g)
2018	270	13	0
2019	298	50	4
2020	218	67	3
2021	380	157	12
2022	345	163	17
2018-2022	1511	450	36

FIGURE 2 | False negative fit results by year and cumulatively. The table shows the total number of patients diagnosed with CRC, FIT tests available on those patients and the number of false negative tests. The graph shows the sensitivity year by year and total (confidence intervals 2018: 77.19%–100%, 2019: 82.45%–97.08%, 2020: 88.14%–95.89%, 2021: 88.00%–95.89%, 2022: 85.40%–94.02% 2018–2022: 89.92%–94.6%).

TABLE 1 | Clinical reason for performing FIT linked to NICE guidelines. Reason for testing was recorded for 387 patients.

Finding	Frequency	Percentage (%)
Changes in bowel habit	199	51.4
Iron deficient anaemia	87	22.5
Unexplained abdominal pain	72	18.6
Unexplained weight loss	39	10.1
Anaemia in the absence of iron deficiency	27	7.0

Note: Not all GPs submitted a reason for performed the FIT and some GPs identified more than one reason, therefore total is >100%.

alone is not to be used to exclude a referral [17]. The data we have presented here support this recommendation further. Although it should be noted that the 36 false negative results identified in patients with confirmed cancer equate to 0.06% of all FIT tests performed in this time period. Therefore, the clinician should ensure that safety netting is in place for their patients, but continue to have confidence that a negative result is likely to be correct.

A change in bowel habit was the most common reason for suspecting colorectal cancer in this patient group. It was more than twice as frequent as the next most common symptom and the leading cause for a clinician to request a FIT test in a patient. The second most commonly reported symptom in these patients with confirmed colorectal cancer was iron deficient anaemia. There is a well-established relationship between iron deficient anaemia and colorectal cancer [18].

It was noteworthy that there were fewer patients diagnosed with colorectal cancer in 2020 than in the preceding 2 years. Also of note was the 28% increase in the number of people diagnosed in 2021 with colorectal cancer than any of the preceding 3 years. We suspect this is due to the COVID-19 pandemic, the UK wide lockdown, and reluctance of people to seek help during this period. It would stand to reason that this is to catch up from those missed in the previous year.

If COVID-19 resulted in fewer people seeking help for their medical conditions this may have resulted in a higher prevalence of undiagnosed cancer in the population. This could have led to a higher pre-test probability of cancer, which in turn may have increased the likelihood of a positive FIT test. The impact of this on our study is unknown.

In our patient population, of the 450 patients who had cancer, 36 had a false negative FIT result. There are many reported factors that could affect the diagnostic accuracy of FIT. Sampling of the faecal sample has been reported to be one of the main sources of erroneous results. It has been hypothesised that if a patient takes a sample from a single point of the faeces or towards the centre of the faeces then this may not give a true reflection of the haemoglobin concentration across the full surface of the faeces [19, 20]. A harmonised procedure for FIT specimen collection has been suggested by a number of previous studies Benton et al [19], Godber et al [20] and Fraser [21].

Another explanation which may affect the diagnostic accuracy of the FIT test is the morphology of the colorectal lesion. Some patients have a flat lesion that does not protrude into the gastrointestinal lumen. Dysplasia which arises from sessile serrated polyps known as sessile serrated adenoma or sessile serrated lesion are much less likely to bleed than some of the more common lesions. Patients with a flat lesion are also more likely to be asymptomatic [22, 23]. Of 36 patients identified in our audit 6 patient's histological reports mentioned sessile masses. FIT is designed to detect haemoglobin in the faeces, it may have to be accepted that these patients will not produce a true positive result because of the nature of their pathology.

Fraser et al [24] suggested that the slower transition time through the digestive tract in women accounts for a higher probability of a false negative FIT in female vs. male patients. In women if the lesion is higher up the gastrointestinal tract the haemoglobin is potentially more likely to deteriorate before it is eliminated and sampled by the patient. Our data found a similar 60/40 male-female ratio in both total patients diagnosed with colorectal cancer and patients with a false negative FIT result. However we recognise that with only 36 false negative patients this is not a large enough number to give certainty that there is not a higher false negative rate in female patients.

A weakness of our study is that it focuses on only those patients with a known diagnosis of colorectal cancer. The outcome of all 56,202 people who had a FIT test during the 5 year period is not known. Potential further work on FIT could look at those biopsies where colorectal cancer was not identified and the use of FIT to detect other pathologies. Therefore our study does not highlight and review the full utility of the FIT test. A negative result is just as important as a positive result in the differential diagnosis of a patient.

SUMMARY TABLE

What Is Known About the Subject

- Colorectal cancer accounts for a significant proportion of UK cancer incidence and deaths.
- Blood in the stool is a common finding in colorectal cancer.
- FIT is a test designed to identify blood in the stool of symptomatic colorectal cancer patients.

What This Papers Adds

- The negative predictive value of FIT in clinical practice may not be as high as initially reported in clinical trials.
- Some insight into the impact of the COVID-19 pandemic on the diagnosis of colorectal cancer.
- Consideration of the accuracy and limitations of FIT.

CONCLUDING STATEMENT

This work represents an advance in biomedical science because it highlights the utility of a test in practice, and sheds light on the impact of COVID-19 on cancer diagnosis rates.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

This study was a clinical audit approved by Hull University Teaching Hospitals NHS Trust, reference number BIOC/SE/2024-25/01. This was an audit of patient outcomes during routine clinical care, all results generated were part of the patient's standard treatment. Written informed consent to participate in this study was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

EC performed the audit and wrote this article. DN, RT, JS, and MH contributed manuscript revision and read. MH was the principle investigator and supervised the work. All authors contributed to the article and approved the submitted version.

FUNDING

The authors declare that no financial support was received for the research, authorship, and/or publication of this article.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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APPENDIX 1 | SNOMED CODES IDENTIFIED IN COLORECTAL CANCER PATIENTS WITH DESCRIPTION.

SNOMED codes	SNOMED description
P206021	Colon neoplasm screening
T67000	Colon, NOS
T67100	Cecum
T67200	Ascending colon
T67400	Transverse colon
T67600	Descending colon
T67700	Sigmoid colon
T68000	Rectum, NOS
T69000	Anus, NOS

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The Use of CD200 in the Differential Diagnosis of B-Cell Lymphoproliferative Disorders

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Background: B-Cell Lymphoproliferative Disorders (B-LPDs) are a group of heterogenous disorders characterised by the accumulation of B-cells in peripheral blood, bone marrow, lymph nodes and spleen. They have a variable disease course and outcome and many share similar features making differential diagnosis challenging. Therefore, accurate diagnosis is fundamental in particular for determining treatment options. Immunophenotyping by flow cytometry plays a crucial role in the diagnosis of B-LPDs. However, overlapping immunophenotyping patterns exist and the use of novel monoclonal antibodies has become increasingly important in immunophenotyping analysis. More recently differential expression of CD200 has been reported in various B-LPDs and that CD200 may improve the differentiation between chronic lymphocytic leukaemia (CLL) and mantle cell lymphoma (MCL). In this study CD200 expression is evaluated in different B-LPDs.

Methods: A total of 100 samples were collected and analysed by immunophenotyping flow cytometry over a period of 1 year (2017–2018), by a panel of monoclonal antibodies including CD200. The percentage of CD200 and its expression intensity was evaluated and compared between different groups of B-LPDs.

Results: All of the 50 cases of CLL expressed CD200 with moderate to bright intensity, 6 MCL cases lacked the expression of CD200. Furthermore, all 5 cases of hairy cell leukaemia (HCL) expressed CD200. Out of all B-LPDs evaluated, CD200 expression in HCL cases was noted to be the brightest. The other 39 cases were not found to be B-LPDs.

Conclusion: CD200 has an important role in differentiating CLL from MCL, HCL has a consistent bright expression of CD200. By adding CD200 to the combinations of markers in routine testing panel, Immunophenotyping by flow cytometry can be an effective tool in the diagnosis of B-LPDs especially in cases with atypical immunophenotyping pattern. Our

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Abbreviations: B-LPDs, B-cell lymphoproliferative disorders; CLL, chronic lymphocytic leukaemia; MCL, mantle cell lymphoma; HCL, hairy cell leukaemia; PB, peripheral blood; FITC, fluorescein isothiocyanate; PE, phycoerythrin; ECD, phycoerythrin-texas red; PC5, phycoerythin cynanin 5; PC7, phycoerythin cynanin 7; MoAbs, fluorescent conjugated monoclonal antibodies; FBC, full blood count; MM, multiple myeloma; AML, acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia; HCLv, hairy cell leukaemia variant.

result support that CD200 can be added to routine testing panel as it is useful in differentiating them.

Keywords: immunophenotyping, flow cytometry, chronic lymphocytic leukaemia, mantle cell lymphoma, hairy cell leukaemia

INTRODUCTION

B-Cell Lymphoproliferative disorders (B-LPDs) consist of a wide range of heterogenous leukaemias and lymphomas characterised by the proliferation of mature B lymphocytes in the peripheral blood, bone marrow and lymphoid tissues [1]. The World Health Organization (WHO) 2017 classification of tumours of haematopoietic and lymphoid neoplasms has classified B-LPDs into different subtypes and their diagnosis is achieved using a multisystem approach based on morphology, immunophenotyping, molecular biology features and cytogenetics [2]. However, there is a significant overlap in some B-LPDs, in particular in their clinical presentation and similar morphological appearance of B cells seen in many. In addition, their clinical outcome differs significantly in terms of treatment options and survival rates. Therefore, an accurate diagnosis is essential and the use of immunophenotyping analysis has become an increasingly important method that is widely used in haematology. Out of B-LPDs, chronic lymphocytic leukaemia (CLL) is the most common leukaemia in Europe and North America [3]. CLL is strongly associated with age and higher incidences are seen in males. There were 3,709 new cases of CLL diagnosed in the UK in 2015 [4].

In 1994 Matutes et al developed a scoring system for the diagnosis of CLL which is based on the immunophenotyping analysis of five markers CD5, CD23, FMC7, CD79b/CD22, and surface membrane immunoglobulin (SmIg). CLL is usually diagnosed when the circulating B lymphocytes exhibit the characteristic immunophenotyping pattern of $CD5^+$, $CD23^+$, FMC7-, CD79b-/CD22- and a weak expression of surface membrane immunoglobulin (SmIg). One score is given for each marker and typical CLL cases are easily identified by a ≥4/5 score. However, problems arise when diagnosing cases that exhibit a non-characteristic immunophenotyping pattern such as atypical CLL cases with a 3/5 score and those non-CLL cases with a 0–2/5 score [5]. Therefore, differential diagnosis between CLL and other B-LPDs remains a big challenge [6], with some studies proposing a new differential diagnosis algorithm [7]. Thus, new and novel markers

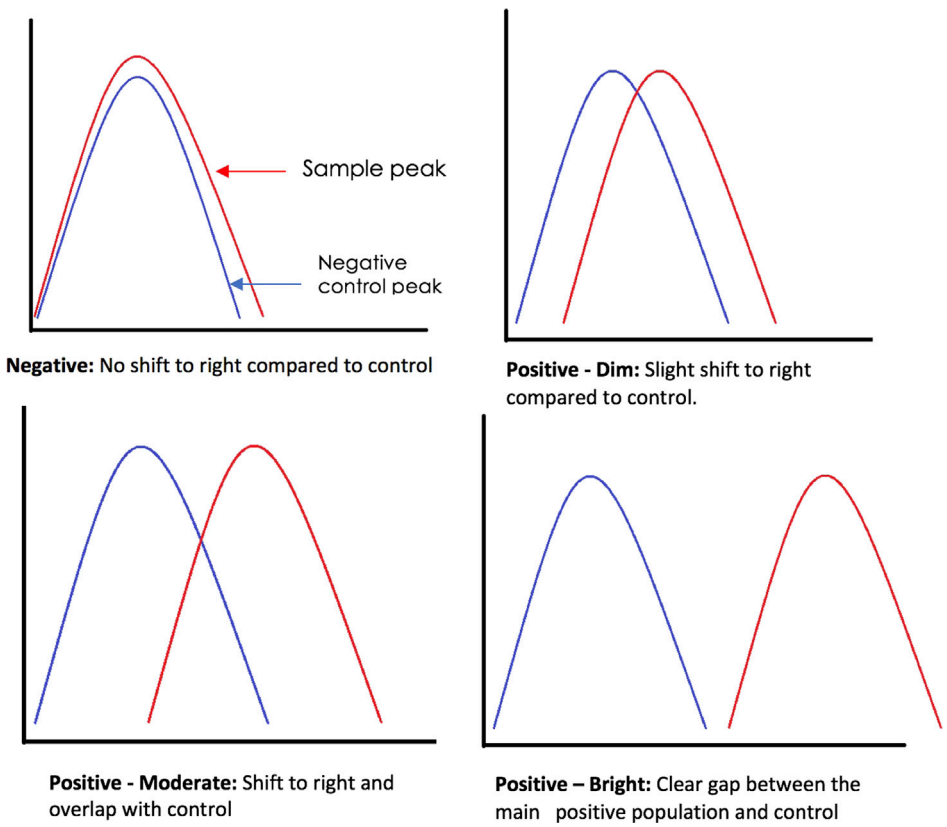

FIGURE 1 | Flow cytometry antigen expression interpretation.

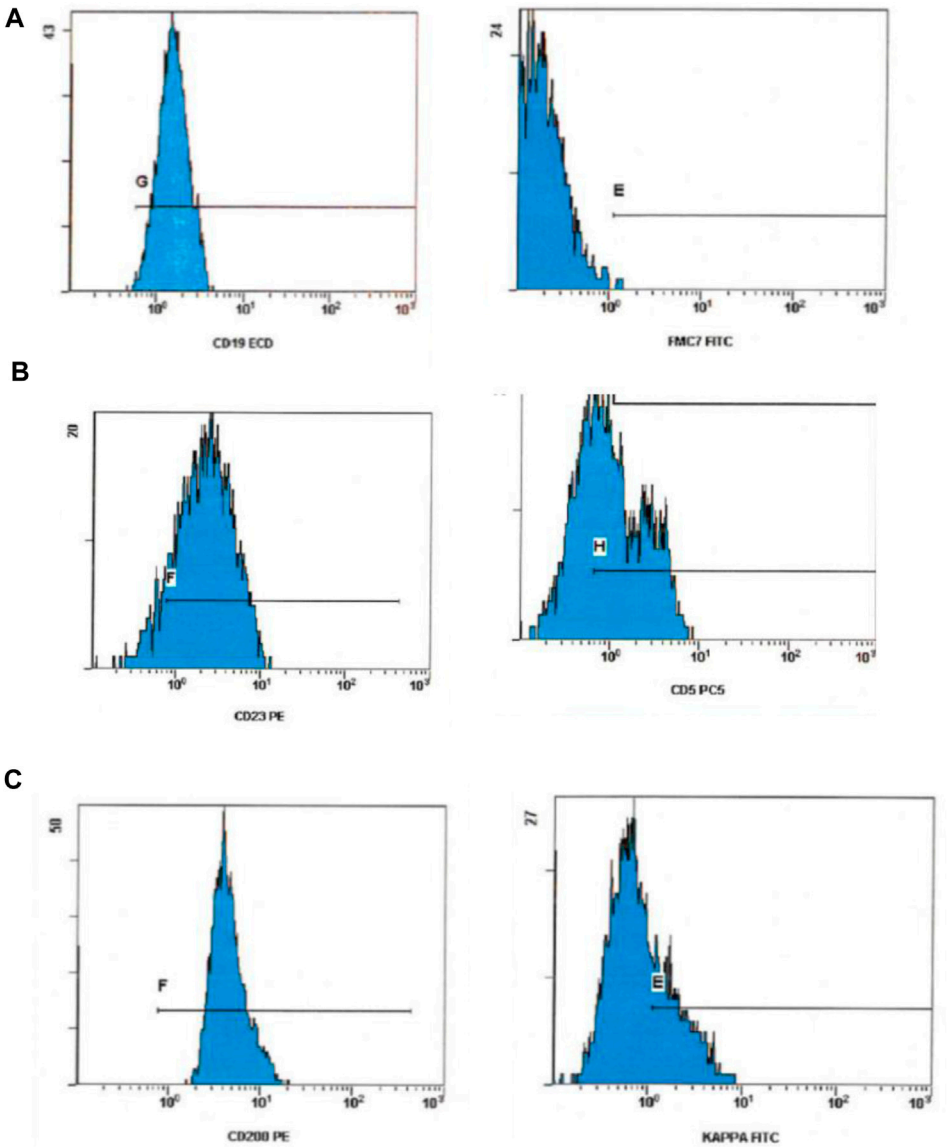

FIGURE 2 | Flow cytometry scatter plot showing expression pattern in CLL [Row **(A)**] CLL with a negative FMC7, [Row **(B)**] positive CD23 and CD5 expression. And, [Row **(C)**] a bright CD200 and dim kappa ex-pression.

are continuously required for immunophenotyping analysis [8]. More recently, it has been discovered that CD200 has a differential expression in B-LPDs such as CLL and MCL which indicated a possible diagnostic value [9].

CD200 (previously known as MRC OX-2) is a membrane glycoprotein belonging to the immunoglobulin superfamily [10], it is encoded by a gene located on chromosome 3q12 [11]. CD200 is expressed on a variety of cell types, including myeloid cells, dendritic cells, neurons, endothelial cells, as well as B and T-lymphocytes [12]. The widely expressed CD200 interacts with the CD200 receptor (CD200R) an inhibitory receptor expressed on monocytes, neutrophils, basophils, macrophages and dendritic cells [13, 14] that plays a vital role in regulating an immune response [15, 16].

The aim of this study was to evaluate the expression of CD200 in various B-LPDs, to determine its usefulness in its differential diagnosis properties in cases already tested at Haematology laboratory, Ysbyty Gwynedd. To support its use in routine Immunophenotyping testing panels. This is a retrospective study, which is part of a service evaluation project.

MATERIALS AND METHODS

Cases tested for lymphoproliferative disease screen at the Specialised Haematology laboratory within the blood sciences department in Ysbyty Gwynedd Hospital between 2017 and 2018 were included in this study. The specialised haematology

service in Ysbyty Gwynedd, Bangor is the only service which provides diagnostic Immunophenotyping by flow cytometry analysis for North Wales, covering three hospital sites (Ysbyty Gwynedd in Bangor, Ysbyty Glan Clwyd in Rhyl and Ysbyty Wrexham Maelor in Wrexham). Most samples are received from Haematology and oncology services within the three hospital sites to confirm the diagnoses of haematological disorders. A total of 100 peripheral blood samples were already analysed at the time of diagnostic request and their data were retrospectively evaluated. The B-LPDs included are CLL (n = 50), MCL (n = 6), HCL (n = 5), and other (n = 39).

Immunophenotyping test is part of the standard diagnostic work up requested by consultant haematologists and GPs that is required to make an accurate haematological diagnosis in which all cases were diagnosed in accordance with the WHO 2017 classification [17] which is based on clinical, morphological and immunophenotyping analysis [2]. The Matutes score was calculated in all cases [18].

This study was approved by the Clinical Effectiveness Department, Ysbyty Gwynedd, Betsi Cadwaladr University Health Board NHS Trust and the Faculty of Medicine, Dentistry and Life Sciences Research Ethics Committee, University of Chester (FREC reference: 1446(1416)/18/HAZ/CMS) [19].

Although this was a retrospective data analysis study on pre-collected and pre-analysed samples. The procedure that was used for analysis is described below.

Flow Cytometry Immunophenotyping Analysis

An Immunophenotyping panel was performed on peripheral blood (PB) samples, using a combination of fluorescein isothiocyanate (FITC), phycoerythrin (PE), phycoerythrin-texas red (ECD), phycoerythin cyanin 5 (PC5), phycoerythrin cynanin 7 (PC7) fluorescent conjugated monoclonal antibodies (MoAb). MoAbs used were sourced from Beckman Coulter, France.

First a full blood count (FBC) was performed on all samples to check that the WBC was $<5 \times 10^9$ on Sysmex XE5000 analyser. A total of 100 µL of EDTA peripheral blood sample was incubated for 15 min in the dark with 10 µL MoAbs. The routine immunophenotyping testing panel in our haematology department for the diagnosis of B-LPDs on PB or BM includes a four-colour combination of MoAbs for CD45, CD2, CD5, CD10, CD19, CD20, CD22, CD23, FMC7, CD43, CD11c, CD103, CD25, CD16/56, Kappa and lambda light chains. In addition, CD200 was included in all cases. A tube containing Ig specific isotype controls for FITC/PE/PC5/ECD were used in all cases, and staining was obtained using the lysed-wash technique, lysed with ammonium chloride lysing buffer and washed in PBS. Tubes were resuspended in PBS, vortexed and analysed using the Beckman Coulter FC500 flow cytometer. Daily quality control procedures were performed using Flow-check and Flow-set beads according to the laboratory standard operating procedures to verify consistent fluorescence intensity during the study.

After completion, data acquisition was performed immediately. For each sample, data from at least 5×10^3 events per tube was acquired. Gating on lymphocytes was achieved on CD45 versus side scatter analysis.

CD200 expression was evaluated by comparison with isotype control and the antigen expression was defined as positive according to the flow cytometric immunophenotyping consensus guidelines (AIEOP-BFM) [20]. CD200 expression intensity was categorised as negative, dim, moderate, and bright.

CD200 is negative when there is no shift to right compared to isotype control tube, dim expression is when there is a slight shift in peak compared to negative control, moderate is when the peak shifts to right and overlaps with the negative control. And bright is where there is a clear gap between the main positive population and the negative control with no virtual overlap (**Figure 1**). An example of scatter plot showing expression seen in CLL is shown in **Figure 2**.

Statistical Analysis

Data collected were analysed with the appropriate statistical tests. Descriptive statistics was presented as number of cases, percentages, Means and SD were calculated. Comparison between groups was performed using non-parametric one-way ANOVA test. All statistical tests were carried out using the statistical package SPSS for Mac version 23 (SPSS Inc., Chicago, IL, USA). Statistical difference was defined when the p-value less than 0.05.

RESULTS

Data from 100 cases tested by flow cytometry Immunophenotyping was retrospectively evaluated. Out of the 100 cases, CLL accounted for the majority of cases, comprising of 50 (50%) cases, of which there were 35 males and 15 females with median age 72µyears. The median haemoglobin (Hb), total white blood count (WBC), lymphocytes count (Lymphs), and platelet count (Plt) were 133.5 g/L, 18×10^9/L, 12×10^9/L, and 209.5×10^9/L respectively. MCL accounted for 6 (6%) of cases, there were 3 males and 3 females with median age 75 years. The median Hb, WBC, Lymphs and Plt count were 126 g/L, 14.8×10^9/L, 9.9×10^9/L, and 136.5×10^9/L respectively. HCL accounted for 5 (5%) cases, of which there were 2 males and 3 females with median age of 56 years. The median Hb, WBC, Lymphs and Plts counts were 126 g/L, 8.3×10^9/L, 1.1×10^9/L, and 155×10^9/L respectively. There were 39 other cases which were found not to have B-LPDs and were therefore, not discussed (see **Tables 1**, **2**; **Figure 3**). The percentage of CD200 positive cells was compared between the three groups, there was a significant statistical difference in % of CD200 between CLL and MCL ($p < 0.001$), and between HCL and MCL ($p < 0.001$). There was no statistical difference in the percentage of CD200 between CLL and HCL ($p > 0.05$.)

DISCUSSION

Studies have reported that CD200 is expressed in haematological malignancies such as multiple myeloma (MM), acute myeloid leukaemia (AML) and acute lymphoblastic leukaemia (ALL) [21,

TABLE 1 | CD200 expression pattern and percentage of positive cells in B-LPDs by Immunophenotyping Flow Cytometry Analysis.

Case	CD200 expression	No. of cases	% Of cells with positive expression of CD200 (mean, median, SD)	Pattern of CD200 expression
CLL	Positive	50	79.9, 81, 13.4	Moderate to Bright
	Negative	0	—	
MCL	Positive	0	—	Absent to dim
	Negative	6	10, 10.5, 9.6	
HCL	Positive	5	64.5, 65, 20.1	Bright
	Negative	0	—	

CLL, chronic lymphocytic leukaemia; MCL, mantle cell lymphoma; HCL, hairy cell leukaemia.

TABLE 2 | Clinical characteristics of CLL, MCL and HCL patients.

	CLL	MCL	HCL
Age (Mean, Median, SD)	70, 72, 10.5	72.5, 75, 6.8	59.6, 56, 12.4
Sex M/F	35/15	3/3	2/3
Total	50	6	5
Haemoglobin (Mean, Median, SD)	131, 133.5, 21	124.5, 126, 8.9	124, 126, 5.8
White Blood cells (Mean, Median, SD)	31.7, 18, 39	21, 14.8, 15	6.3, 8.3, 3.6
Lymphocytes (Mean, Median, SD)	25, 12, 38	15.9, 9.9, 13.8	2.1, 1.1, 2.5
Platelets (Mean, Median, SD)	220, 209.5, 103	149.8, 136.5, 64.9	153, 155, 70

CLL, chronic lymphocytic leukaemia; MCL, mantle cell lymphoma; HCL, hairy cell leukaemia.

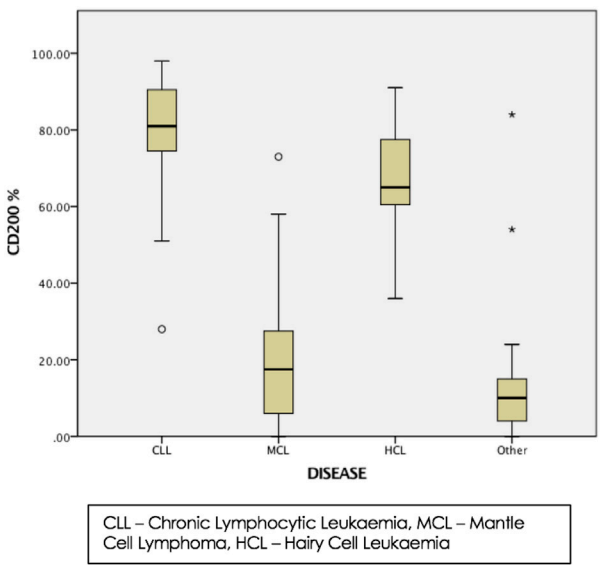

FIGURE 3 | Box plot showing the % of cells with positive expression for CD200 in B-LPDs. The lines inside the box and bars represent median and interquartile ranges. Asterisks and circles represent CD200 values not included between the vertical lines (outliners).

22]. In MM and AML, CD200 expression is used as a prognostic markers as high levels of expression are associated with poor prognosis [23, 24]. CD200 expression was then reported for CLL [22]. Since then, studies have shown that CD200 was expressed differently between CLL and mantle cell lymphoma (MCL) it is consistently expressed in CLL whereas MCL lack the expression of CD200 [9, 25–27]. In addition, CD200 is also expressed in hairy cell leukaemia (HCL) [28].

In this study, CD200 expression was retrospectively evaluated in samples pre-analysed as part of routine immunophenotyping testing at Ysbyty Gwynedd, Bangor, to validate its usefulness in differentiating between different B-LPDs.

CD200 Expression in CLL

There is no single marker for the definitive diagnosis of CLL by Immunophenotyping and in general, the diagnosis of CLL is easily achieved in the presence of the characteristic immunophenotyping pattern ($CD5^+$, $CD23^+$, FMC7-, CD22-/CD79b- and weak expression of SmIg) which is based on the Matutes scoring system [29]. However, problems arise in both diagnosing and differentiating CLL from other B-LPDs in cases where immunophenotyping features are not typical such as cases with Matutes score of 2–3/5 as a study showed that in using this system, they found that 92% of CLL cases score 4 or 5, score 3 is seen in about 6% of CLL cases and 2% of CLL cases score 1 or 2 [30] which results in additional markers being analysed to aid in the diagnosis and further testing is usually the case to make a definitive diagnosis.

We have confirmed previous studies that CD200 is consistently expressed in CLL [9, 31-33], with 50/50 (100%) of CLL cases in this study expressing CD200 (mean % of cells with positive expression of CD200 = 79.9%). A similar finding was also reported where CD200 expression was observed in 100% of CLL in similar studies [9, 25, 34, 35].

CD200 Expression in CLL and MCL

MCL cases in this study lacked CD200 expression. Confirming previous studies [9, 21, 25, 34]. The difference in CD200 expression between CLL and MCL cases was found to be statistically significant.

For many years, CD23 has been a reliable marker that is widely used to differentiate between B-LPDs, in particular CLL and MCL as it is positive in CLL and negative in MCL [36, 37]. However,

TABLE 3 | CD200 expression reported in similar studies.

Study	Total No of cases	CD200 expression		
		CLL cases	MCL cases	HCL cases
[35]	50	30/30 (100%)	—	5/5 (100%)
Poongodo R et al., 2018	77	54/54 (100%)	1/6 (16%)	5/5 (100%)
[28]	160	98/98 (100%)	0/24 (0%)	6/6 (100%)
Gorczynski et al., 2017	70	45/45 (100%)	0/14 (0%)	—
Mason et al., 2017	79	—	—	34/34 (100%)
Fan L et al., 2015	374	268/271 (98.8%)	1/31 (3%)	2/5 (40%)
El-Sewefy DA et al., 2014	40	30/30 (100%)	0/10 (0%)	—
[53]	159	56/56 (100%)	0/14 (0%)	13/13 (100%)
[54]	364	119/119 (100%)	3/61 (5%)	7/7 (100%)
[52]	180	27/27 (100%)	0/14 (0%)	10/10
[34]	107	19/19 (100%)	0/4 (0%)	—

CLL, chronic lymphocytic leukaemia; MCL, mantle cell lymphoma; HCL, hairy cell leukaemia.

CD23 as a single marker is not sufficient to make a definitive distinction between CLL and MCL. In addition, in occasional cases, it has been reported that CLL have weak or no expression of CD23 [26, 38], and a minority of MCL cases can express CD23 as studies have shown that approximately 20%–30% of MCL cases can have a positive CD23 expression [26, 39–41].

Other antigens that are used to distinguish between CLL and MCL include FMC7, which is usually negative in CLL and expressed in MCL [42]. However, similar to CD23, studies have reported positive expression of FMC7 is seen in about 12% of CLL cases [36, 43–45] and 90%–100% of MCL cases [36, 42], making cases with an unusual phenotype difficult to diagnose.

The differentiation between MCL and CLL is crucial as MCL is characterised by an aggressive clinical course with continuous relapse and poor prognostic outcome [46–48], and sometimes clinicians rely on other tests such as testing for Cyclin D1 and/or the detection of chromosomal translocation t(11; 14) for a definitive diagnosis which uses techniques such as polymerase chain reaction (PCR) and Western blot which are both costly, time consuming and not available in all Haematology laboratories. In addition, there have been reported cases of MCL which lack the positivity of Cyclin D1 [49, 50]. Therefore, we believe in cases were diagnosis is difficult to achieve, the addition of CD200 as an extra marker is useful in differentiating between CLL and MCL as Immunophenotyping by flow cytometry is not difficult, is fast and less expensive. In this study, CD200 was consistently expressed in CLL cases and MCL cases lacked the expression of CD200. CD200 was found to be excellent marker in differentiating between CLL and MCL.

CD200 Expression in CLL and HCL

In this study, CD200 expression was observed in all HCL cases 100% (5/5) the mean % of cells with positive expression of CD200 = 64.5%, which was a similar finding reported in other studies [25, 35, 51]. Also, the HCL cases in this study showed the brightest intensity for CD200 expression out of all cases. Bright CD200 expression in HCL cases has also been reported in similar studies [25, 51–54]. Others have confirmed CD200 expression in HCL by Immunohistochemistry analysis on formalin fixed paraffin embedded tissue sections of bone marrow biopsy and lymph nodes [21].

The characteristic immunophenotyping feature of HCL is the expression of CD19, CD20, CD22, CD25, CD11c, CD103 and SmIg [17, 55, 56]. Also, to differentiate HCL from CLL, HCL is usually CD23 negative and FMC7 positive whereas CLL has the opposite phenotype [29]. Discrepancy in the classic immunophenotyping pattern in HCL has been described with one study demonstrated positive expression of CD23 in about 17% of HCL cases, the lack of CD25 expression in 3% of HCL cases, and lack of CD103 in 6% of HCL cases [55]. Additionally, the markers expressed in HCL such as CD25, CD103 and CD123 are often not present in the initial immunophenotyping testing panel for the diagnosis of LPDs, as such an additional panel using extra markers might have to be set up. By adding CD200 to the initial routine testing panel, the bright expression of CD200 might raise a suspicion that HCL is likely.

It has been reported that CD200 is not expressed in a variant form of HCL (HCLv) [28, 52]. Although no cases of HCLv was included in this study. HCLv has a similar clinical and morphological features to HCL [57, 58] but patients are resistant to HCL therapy and require special treatment options [59, 60]. Therefore, differentiating between the two is important.

CD200 not only aids in confirming a diagnosis of HCL, but also distinguishes HCL from HCLv. We believe that adding CD200 to the initial immunophenotyping testing panel would be significantly beneficial.

Overall, the findings of this study agree with previous studies confirming the expression of CD200 in CLL, HCL and its lack of expression in MCL. The expression of CD200 in other similar studies is summarised in **Table 3**.

This study has its limitation, first the sample size was relatively small limited to 100 samples, as this was a retrospective data analysis for samples tested over a 1 year period, this could be improved by extending the period for the retrospective collection of data for about two or three years. Also, during the 1 year, the cases diagnosed were limited to CLL, HCL and MCL so may be other B-LPDs would have been diagnosed if the study was extended to allow more cases to be included.

The majority of studies have focused on the use of CD200 to differentiate between CLL and MCL, limited reports on other B-LPDs are available. Thus, more studies are needed to include cases for other B-LPDs. In particular, it would be useful to analyse CD200 expression in those MCL cases that are negative for cyclin

D1. Also, there are a limited number of reports which have evaluated the expression of CD200 between HCL and HCLv and used a small number of cases. Therefore, the role of CD200 in differentiating between HCL and HCLv could be investigated further. Although many have investigated CD200 expression by flow cytometry, some reports have also demonstrated its usefulness in immunohistochemistry [21]. Further studies comparing CD200 expression using the two methods could be beneficial.

In addition to its diagnostic value, CD200 has been shown to have a prognostic role in diseases such as in acute lymphoblastic leukaemia [61]. Its expression has been associated with an unfavourable prognostic outcome in AML and MM [62]. And in CLL, low expression of CD200 has been associated with predicting shorter time needed for treatment [63].

There has been some work into anti-CD200 targeted therapy and that anti-CD200 can supress tumour cells and restore tumour immune control in an animal model [64]. This has led to the development of humanised monoclonal anti-CD200 antibody ALXN600 used in phase I/II clinical trial (NCT00648739) for patients with CLL and MM with only mild to moderate side effects reported [65]. The overexpression of CD200 has also been involved in the pathogenesis of various tumours including renal, colon, testicular head and neck carcinoma [66–68]. Thus, targeting CD200 may be hopeful for the future in a large number of malignancies.

To conclude, there is no single marker for the definite diagnosis of CLL by Immunophenotyping and although the Matutes score has been the basis of the diagnosis of CLL, sometimes relying on the markers used in the score alone is not sufficient in the differential diagnosis with some cases relying on further testing such as Immunohistochemistry (IHC), cytogenetics fluorescence *in situ* hybridisation (FISH) testing which are time consuming, expensive and are not always available in all haematology laboratories and require sending to other centres with additional samples requested. Since Immunophenotyping testing is already performed for the diagnosis of B-LPDs cases, and the addition of further markers will not only be time saving but also economical as no additional diagnostic sample would be required. Therefore, the addition of an extra marker such as CD200 is of a significant diagnostic value.

Our results confirm that CD200 is a valuable marker in confirming the diagnosis of CLL and HCL, adding CD200 to the routine testing panel will aid in differentiating between CLL and MCL in cases with overlapping immunophenotyping results. Therefore, it should be included in routine testing Immunophenotyping panels to aid in differentiating between various B-LPDs. In the future, CD200 could be a possible therapeutic target especially in patients with a bright CD200 expression as seen in HCL and CLL.

SUMMARY TABLE

What is Known About This Topic?

- There is no single marker for the definite diagnosis of B-cell Lymphoproliferative disorders.
- CD200 is expressed in B-cell Lymphoproliferative disorders such as CLL and HCL.

What This Work Adds

- It expands the understanding of CD200 expression in B-cell Lymphoproliferative disorders.
- It confirms the importance of CD200 in diagnosing CLL and HCL as well as differentiating between various B-cell Lymphoproliferative disorders.
- It gives further support to include CD200 in routine Immunophenotyping testing panels to aid in the diagnosis of B-cell LPDs.

This work represents an advance in biomedical science because it shows the importance of CD200 and its significant diagnostic value when used in immunophenotyping panels for the diagnosis of B-cell Lymphoproliferative disorders.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

This study was approved by the Clinical Effectiveness Department, Ysbyty Gwynedd, Betsi Cadwaladr University Health Board NHS Trust and the Faculty of Medicine, Dentistry and Life Sciences Research Ethics Committee, University of Chester [FREC reference: 1446(1416)/18/HAZ/CMS]. Written informed consent to participate in this study was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

HA-Z: Performed data analysis and wrote the article, SH: critical revision of the article. All authors contributed to the article and approved the submitted version.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Prognostic Role of CD68$^+$ and CD163$^+$ Tumour-Associated Macrophages and PD-L1 Expression in Oral Squamous Cell Carcinoma: A Meta-Analysis

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Background: Oral squamous cell carcinoma (OSCC) is a common malignant cancer in humans. An abundance of tumour associated macrophages (TAMs) create an immunosuppressive tumour microenvironment (TME). TAM markers (CD163 and CD68) are seen to serve as prognostic factors in OSCC. PD-L1 has seen to widely modulate the TME but its prognostic significance remains controversial. The aim of this meta-analysis is to evaluate the prognostic role of CD163$^+$, CD68$^+$ TAMs and PD-L1 in OSCC patients.

Methods: Searches in PubMed, Scopus and Web of Science were performed; 12 studies were included in this meta-analysis. Quality assessment of included studies was performed according to REMARK guidelines. Risk of bias across studies was investigated according to the rate of heterogeneity. Meta-analysis was performed to investigate the association of all three biomarkers with overall survival (OS).

Results: High expression of CD163$^+$ TAMs were associated with poor overall survival (HR = 2.64; 95% CI: [1.65, 4.23]; $p < 0.0001$). Additionally, high stromal expression of CD163$^+$ TAMs correlated with poor overall survival (HR = 3.56; 95% CI: [2.33, 5.44]; $p < 0.00001$). Conversely, high CD68 and PD-L1 expression was not associated with overall survival (HR = 1.26; 95% CI: [0.76, 2.07]; $p = 0.37$) (HR = 0.64; 95% CI: [0.35, 1.18]; $p = 0.15$).

Conclusion: In conclusion, our findings indicate CD163$^+$ can provide prognostic utility in OSCC. However, our data suggests CD68$^+$ TAMs were not associated with any prognostic relevance in OSCC patients, whereas PD-L1 expression may prove to be a differential prognostic marker dependent on tumour location and stage of progression.

Keywords: macrophages, oral cancer, CD68, PD-L1, CD163

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is a common malignant neoplasm (80%–90%) of the oral cavity, derived from the head and neck region of the body. It is associated with the common risk factors of smoking and alcohol consumption (1, 2). Contributing the highest incidence and mortality rate in both males and females, there were 354,864 new cases and 177,384 deaths worldwide in 2018 and was the leading cause of mortality in Central Asia (3). Whilst there is an improvement in advancing therapies such as surgery and chemotherapy, the 5-year survival rate remains 50% in various countries over the past 3 decades (4). This insufficient improvement in prognosis could be explained by the lack of consideration of immunological parameters in prognostic classification and treatment of OSCC (5). Moreover, poor prognosis in OSCC may be a result of its aggressive local invasion and metastasis, leading to an uncontrollable recurrence (6).

Metastasis is achieved through the interaction of tumour cells and the surrounding tumour microenvironment (TME) (7). This TME plays a critical role in tumourigenesis, tumour progression, invasion and tumour tissue infiltration by tumour-associated macrophages (TAMs) (8). TAMs are abundant in both the tumour and tumour stroma, playing a significant role in cancer progression (9, 10). MCP-1 (Monocyte chemoattractant protein-1 or CCL2) plays a role in recruiting and attracting TAMs to tumour sites (11, 12). These TAMs may exhibit either of two functional phenotypes, M1 or M2, dependent on cytokine, chemokine, chemokine receptor and other regulator expression (13). M1 TAMs exhibit pro-inflammatory and anti-tumoural properties, mediated by IL-12, TNF-α, IFN-γ, and stimulate strong Th1 IFNγ-driven cell mediated responses resulting in tumouricidal function. M2 TAMs are generally anti-inflammatory and pro-tumoural, expressing IL-10, IL-13, MR (mannose receptor) and are capable of inducing humoral Th2-driven cytokine responses, secreting IL-4, IL-13 and high levels of chemokines and growth factors such as VEGF, TGF-β, FGF and uPA, promoting angiogenesis, immunosuppression, tumour invasion and metastasis (14, 15).

TAM polarisation to distinct M1 and M2 subsets however, remains unclear, as recent evidence suggests functional plasticity and the ability to repolarise from one phenotype to the other (16). Human ovarian cancer TAMs have been observed to repolarise from M2 to M1-like phenotype suppressing levels of CCL18, MMP9 and VEGF when exposed to IFN-γ (17). TME TAMs have been shown to favour tumourigenesis, tumour survival and angiogenesis (18). This role in the TME however, is controversial; in colorectal cancer, for example, TAMs exhibit pro-inflammatory anti-tumour effects, leading to a favourable prognosis (19, 20). This may be explained by these M1 TAMs inducing the secretion of galectin-3 in human colon cells which further induces TAM infiltration and release of the pro-inflammatory cytokines, IL-1β and TNF-α, causing a strong anti-tumour response (20). Nevertheless, whilst TAMs may exhibit either phenotype, studies recognise TAMs to be predominantly of the M2 phenotype and correlate with a poor prognosis (15, 21).

TAMs may thus serve as potential biomarkers for the prognosis and therapeutic targeting of several cancers, particularly OSCC. Interestingly, over 80% of studies reveal a high number of TAMs correlates with poor patient prognosis (21). Investigations have shown CD163 (M2 macrophage class B scavenger receptor) (22), as a biomarker for macrophage activation in lung, breast and hepatocellular carcinoma (23-25). Recently, overexpression of M2-like $CD163^+$ TAMs in head and neck squamous cell carcinoma (HNSCC) patients, revealed a poor clinical prognosis in both overall survival (OS) and progression-free survival (PFS) (26). CD163 functionality involves eradicating and endocytosing the haemoglobin/haptoglobin complex, thereby protecting tissues from oxidative damage (27). Used as a biomarker for M2c deactivated macrophages, it presents both anti-inflammatory and pro-tumoural functions (28).

Monocyte/macrophages, specifically M2 macrophages, abundantly express CD68, a glycosylated type I transmembrane glycoprotein belonging to the LAMP (lysosomal-associated membrane proteins) family (29). Its primary function is poorly understood, but as a class D scavenger receptor, it plays a role in promoting phagocytosis, clearing cellular debris and mediates recruitment/activation of macrophages (30). In contrast, CD68 is considered to be a pan-macrophage marker expressed by both M1 and M2 subsets, derived from anti-CD14-purified peripheral blood monocytes (31). This may explain observations where overexpression of $CD68^+$ TAMs was associated with poor overall survival and disease-free survival (DFS) in breast cancer patients (32), whereas conversely, high $CD68^+$ TAM expression conferred a longer overall survival and disease-free survival in hepatocellular carcinoma patients (33). Therefore, its prognostic relevance to OSCC needs clarification.

PD-L1 (programmed death ligand-1 also known as CD274, B7-H1) is a cell surface type I glycoprotein expressed on antigen-presenting cells (APCs) and located in dendritic cells and macrophages. Belonging to the B7 family, it is a co-inhibitory ligand which binds PD-1 (programmed death receptor-1). PD-1 functions as a T-cell checkpoint protein, regulating T-cell suppression (34). PD-1 is a member of CTLA-4 (cytotoxic T lymphocyte-associated protein 4) family, primarily expressed by cytotoxic T cells (Tc), which predominate anti-tumour responses. PD-1 ligation suppresses T-cell function *via* an inhibitory signal involving SHP-2 which inhibits CD28-mediated PI3K and Akt activity (35). In various cancers, PD-1 can be expressed on tumour infiltrating lymphocytes (TILs), where $CD4^+$ and $CD8^+$ TILs exhibit an increased PD-1 expression on Treg and Tc, effectively resulting in Treg-mediated immunosuppression and Tc anergy/loss of CTL function (36). In order to maintain homeostasis, PD-1/PD-L1 induces immune tolerance and effectively suppresses excessive tissue inflammation and autoimmune disease. In tumours, however, binding of PD-L1 to its PD-1 receptor on activated T cells results in T-cell suppression and immune escape by inhibiting perforin/granzyme production, suppressing IL-2 and IFN-γ production and promoting apoptosis, effectively inducing tumour growth (37). Several studies investigated the relationship between TAM

PD-L1 expression and cancer patient prognosis. High PD-L1 expression revealed a poor clinical prognosis in malignant pleural mesothelioma (MPM) and renal cell carcinoma patients (38, 39), whereas other studies reached controversial and inconsistent conclusions. Conversely, high PD-L1 expression was associated with longer overall and disease-free survival (40, 41). Therefore, its prognostic relevance needs further clarification.

This study aims to investigate the prognostic role of $CD68^+$, $CD163^+$ TAMs and PD-L1 expression in OSCC, through a meta-analysis of the current literature. Furthermore, the prognostic role of these biomarkers was investigated in different sub-locations in OSCC (tumour versus stroma). This study hypothesised a high expression of $CD68^+$, $CD163^+$ TAMs and PD-L1 would lead to worse survival in OSCC patients, whereas concluded that $CD163^+$ TAMs located in both tumour and stroma were predictive of a poor prognosis in OSCC and that PD-L1 may prove to be indicative of a positive outcome in these patients.

MATERIALS AND METHODS

Search Strategy

In order to identify potential studies, a systematic search was conducted on the following online databases: PubMed, Scopus and Web of Science. Two Boolean operators (AND, OR) were used to select specific keywords. The following terms include: (macrophage OR TAM OR "tumour-associated macrophage" OR CD68 OR CD163) AND ("oral cancer" OR "oral squamous cell carcinoma" OR OSCC) AND (survival OR prognosis OR mortality OR death) AND (PD-L1 OR programmed death ligand 1 OR PDL1 OR B7-H1). Title and abstracts were screened based on inclusion and exclusion criteria (Refer to eligibility criteria section below). After inspecting full texts, the final predetermined articles were selected.

ELIGIBILITY CRITERIA—INCLUDED AND EXCLUDED STUDIES

Studies that had met the following inclusion criteria were included in the meta-analysis: 1) English language publication. 2) Studies that reported the prognostic significance and role of CD163, CD68 and PD-L1 in OSCC 3) Studies analysing the protein level expression of CD163, CD68 and PD-L1 in clinical analysis such as immunohistochemistry (IHC) sections in OSCC. 4) Evaluate the association of CD163, CD68 and PD-L1 and patient prognosis according to the following parameters: overall survival (OS). 5) Provided sufficient survival data which included only hazard ratio (HR) with 95% confidence interval (Cl), *p*-value *P*) alongside Kaplan Meier survival graphs. Studies that had less than 30 patients and did not meet the parameters were excluded from the meta-analysis.

Data Extraction (Outcomes)

Included studies that met the criteria had their extracted data in accordance to: name of first author, year of publication, region of study, sample size, age, type of biomarkers used (CD163, CD68 or both and PD-L1), stage of cancer (TNM stage), location of tumour analysed, follow-up, cut-off values (threshold for prognostic factor and corresponding outcome based on high-risk and low-risk groups) and univariate and/or multivariate analysis outcomes to extract HR and 95% Cl for OS. Articles providing survival data are visualised in Kaplan-Meier curves.

Risk of Bias

To determine the risk of bias for each study, a quality assessment was conducted in accordance with the REMARK (Reporting Recommendations for Tumour Marker Prognostic Studies) (42). The risk of bias consists of six components: 1) samples, 2) clinical data of the group, 3) immunohistochemistry, 4) prognostication, 5) statistics and 6) prognostic factors. Each component was considered as: sufficient, insufficient or N/A (no description). The assessment scores for each study are shown in **Table 1** according to REMARK assessment criteria guidelines presented in **Supplementary Table S2**.

Quantitative Data Analysis

RevMan (Review Manager) 5.4.1 was used to extract and construct quantitative data for the meta-analysis. Hazard ratios (either univariate or multivariate estimates), 95% confidence intervals (95% Cl) and *p*-value *P*) were extracted from the included studies. This data was constructed in forest plots with all in a random effect model. HRs for all immune biomarkers were sorted in a high vs. low direction. If HR estimates are reported in the opposite direction, HR and 95% Cl values were inverted. An HR >1 corresponds to worse survival in the group with high $CD68^+$, $CD163^+$ TAMs or PD-L1 expression. Estimated values of CD163, CD68 and PD-L1 expression were performed based on survival variables such as overall survival (OS). Other survival rates including disease-free survival (DFS) and disease-specific survival (DSS) was not included due to insufficient data. *p*-value lower than 0.05 was considered statistically significant. Heterogeneity (I^2) is assessed and classified by Higgins index with: low heterogeneity (25%), medium heterogeneity (50%) and high heterogeneity (70%) (43).

RESULTS

Study Selection and Study Results

Searches revealed 1881 records from commonly used databases (PubMed, Scopus and Web of Science) which justified the best array of literature. A total of 207 records were screened by title and abstract. Of these 207 articles, 177 were excluded due to providing insufficient data. 30 articles met the initial assessment of the inclusion criteria. Eventually, 12 studies were considered for inclusion in the meta-analysis (**Figure 1**) analysing data from 1373 patients (see also **Supplementary Table S1**). Three studies analysed $CD68^+$ in the stroma and intra-tumour location of OSCC. Three studies analysed $CD163^+$ TAM in the stroma. 12 of the included studies were predominately performed in

TABLE 1 | Quality assessment of studies included in the meta-analysis according to REMARK guidelines.

Author/year [References]	Country	Samples	Clinical data	Immunohistochemistry	Prognostication	Statistics	Prognostic factors
Fujii/2012 (44)	Japan	S	S	S	S	S	S
Fujita/2014 (45)	China	S	S	S	I	I	S
Wang/2014 (46)	China	S	S	S	S	S	S
Matsuoka/2015 (47)	Japan	S	S	S	S	S	S
Takahashi/2017 (48)	Japan	S	S	S	S	S	S
Ni/2015 (49)	China	S	S	S	S	S	S
Fang/2017 (50)	China	S	S	S	S	I	S
Kikuchi/2021 (51)	Japan	S	S	S	I	S	S
Lin/2015 (52)	Taiwan	S	S	S	S	I	S
Kogashiwa/2017 (40)	Japan	S	S	S	S	S	S
Ahn/2017 (53)	South Korea	S	S	S	I	S	S
Lenouvel/2021 (54)	Spain	S	S	S	S	I	S

Included studies scaled: S, sufficient; I, insufficient; N/A, no description.

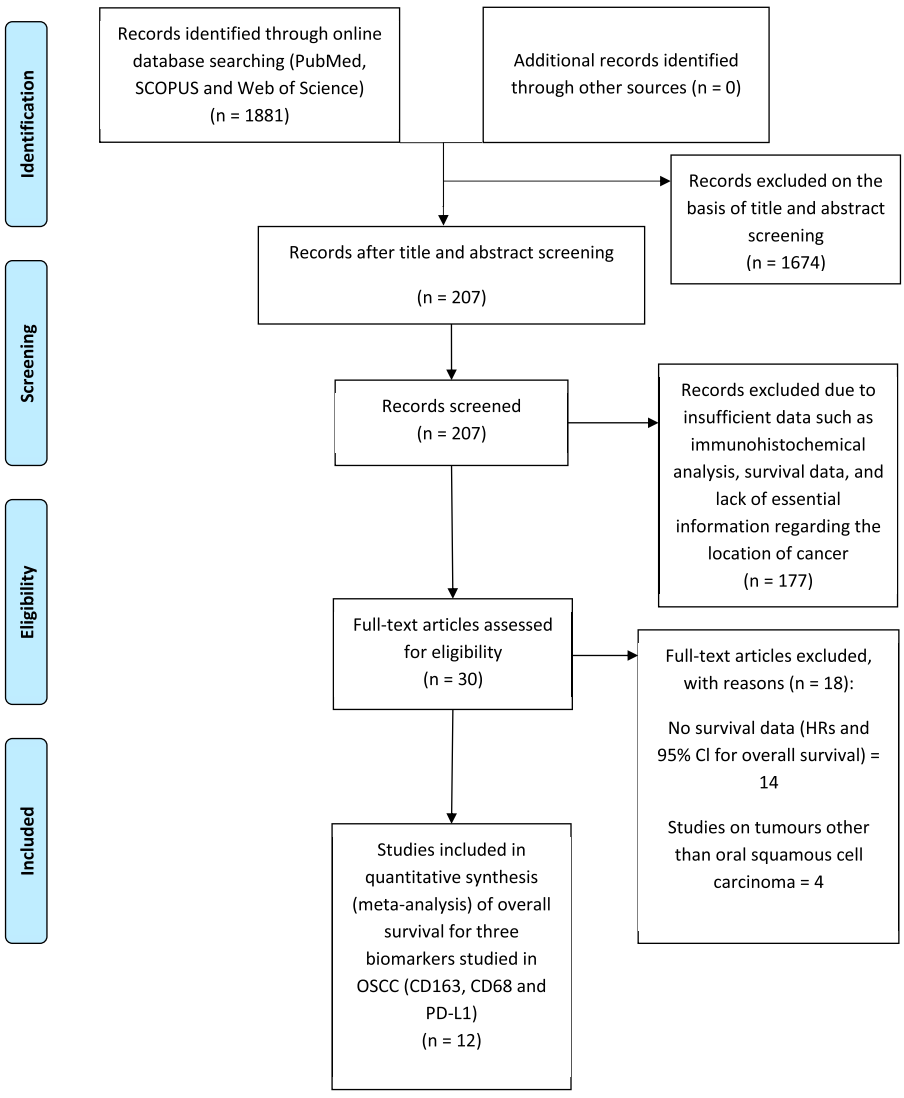

FIGURE 1 | PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analysis) flow diagram of the study selection process for meta-analysis summarising literature searching, screening and assessment of eligibility of identified studies.

TABLE 2 | Main characteristics of included studies in meta-analysis.

Author/year [References]	Region	No. of participants	Age (mean, Range)	Location of tumour analysed	Stage
Fujii et al., 2012 (44)	Japan	108	66.4, 23–93	Tumour Stroma	I-IV
Fujita et al., 2014 (45)	China	50	68.6, 48–93	Invasive front	I-IV
Wang et al., 2014 (46)	China	298	53, 21–78	Tumour Stroma	I-IV
Matsuoka et al., 2015 (47)	Japan	60	68.9, 33–87	Tumour Stroma at invasive front	I-IV
Takahashi et al., 2017 (48)	Japan	73	69, 36–92	Tumour Stroma	I-IV
Ni et al., 2015 (49)	China	91	55, 20–78	Normal OSCC tissue, tumour nest and tumour stroma	I-IV
Fang et al., 2017 (50)	China	78	60, 24–82	Tumour stroma, tumour epithelial, advancing tumour margin	I-IV
Kikuchi et al., 2021 (51)	Japan	103	70, 30–92	Tumour Stroma, Intra-tumoural compartment	I-IV
Lin et al., 2015 (52)	Taiwan	305	N/A	Normal OSCC tissue	I-IV
Kogashiwa et al., 2017 (40)	Japan	84	68, 20–92	N/A	I-IV
Ahn et al., 2017 (53)	South Korea	68	57.7, 23–84	Normal OSCC tissue	I-IV
Lenouvel et al., 2021 (54)	Spain	55	66.8, 42–87	Tumour Stroma	I-IV

N/A, not reported.

TABLE 3 | Data extraction from included studies related to outcomes in meta-analysis.

Author/year [References]	Biomarker	Follow-up (months)	Cut-off point	Univariate or multivariate analysis	Overall survival (HR (hazard ratio), 95% cl)
Fujii et al., 2012 (44)	CD163	N/A	Median, 1.6 HPF (high pass filter) (CD163)	Multivariate	2.64, 1.02–6.80
Fujita et al., 2014 (45)	CD163	N/A	Median	Multivariate	4.53, 0.75–27.36 (Estimated)
Wang et al., 2014 (46)	CD163	61.5 (Median)	Median	Multivariate	3.56, 1.67–7.59
Matsuoka et al., 2015 (47)	CD163	N/A	Median, 3.2 HPF (CD163)	Multivariate	2.30, 0.65–8.10
Takahashi et al., 2017 (48)	CD68, CD163	30.5 (Median)	Median, 204 ±200 (CD68), 64 ± 55 (CD163)	Univariate (CD68) Multivariate (CD163)	1.11. 0.34–3.70 (CD163) 2.33, 1.00–5.45 (CD68)
Ni et al., 2015 (49)	CD68	N/A	≥75%	Univariate	1.39, 0.28–6.89
Fang et al., 2017 (50)	CD68	48 (Median)	Mean	Multivariate	0.73, 0.43–1.31
Kikuchi et al., 2021 (51)	CD68, PD-L1	40.8 (Median)	Median ≥1 and ≥20	Univariate (CD68) Univariate (PD-L1)	0.84, 0.31–2.26 (CD68) 0.50, 0.18–1.39 (PD-L1)
Lin et al., 2015 (52)	PD-L1	45.6 (Mean)	N/A	Univariate	1.21, 0.89–1.64
Kogashiwa et al., 2017 (40)	PD-L1	40.6 (Mean)	Mean	Multivariate	0.26, 0.10–0.65
Ahn et al., 2017 (53)	PD-L1	44.3 (Mean)	N/A	Univariate	0.32, 0.11–0.93
Lenouvel et al., 2021 (54)	PD-L1	56 (Median)	5% TPS (tumour proportion score)	Univariate	0.58, 0.14–2.45

N/A, not reported

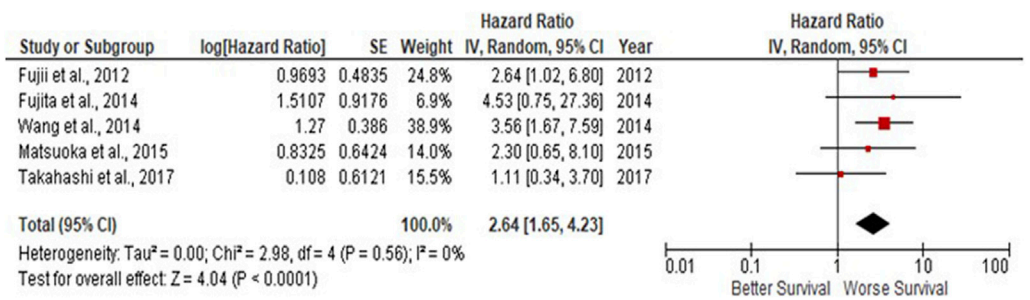

FIGURE 2 | CD163$^+$ TAMs are associated with poor overall survival in OSCC Forest plot reveals Hazard ratios (HRs) and 95% CI for the association of CD163$^+$ TAMs and overall survival (OS) in OSCC patients. Red square represents hazard ratio for each study, horizontal lines represent 95% confidence intervals and vertical line represents line of no effect. Black diamond represents the mean weighted overall hazard ratio among all studies (pooled estimate). An HR >1 illustrates a higher risk of death or progression associated with high CD163$^+$ TAM expression. Forest plot reveals statistical significance between high CD163$^+$ TAMs expression and OS in OSCC patients ($p < 0.0001$). Heterogeneity equates to 0% and results are conducted in a random-effect model.

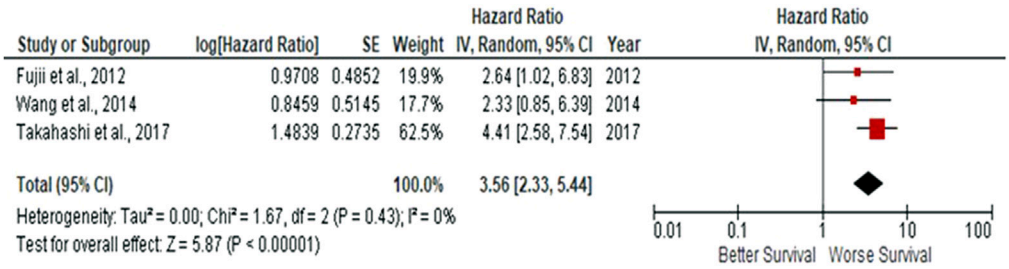

FIGURE 3 | Stromal-located CD163$^+$ TAMs are also associated with poor overall survival in OSCC Forest-plot reveals hazard ratios (HRs) and 95% CI in accordance to stromal localisation of CD163$^+$ TAMs in OSCC samples. Red square represents hazard ratio for each study, horizontal lines represent 95% confidence intervals and vertical line represents line of no effect. Black diamond represents the mean weighted overall hazard ratio among all studies (pooled estimate). An HR >1 illustrates a higher risk of death or progression associated with high stromal CD163$^+$ TAMs. Forest plot reveals statistical significance between high CD163$^+$ TAMs expression in accordance to stromal localisation in OSCC samples ($p < 0.00001$). Heterogeneity equates to 0% and results are conducted in a random-effect model.

Asia and one in Europe. The main characteristics of eligible studies and data extraction are shown in **Tables 2**, **3**.

CD163$^+$ TAMs are Associated With Poor Prognosis in OSCC

Due to observations that M2-like CD163$^+$ TAMs were associated with poor prognosis in HNSCC, breast, gastric, colorectal and hepatocellular cancers, this study investigated whether CD163$^+$ TAMs could also be adopted as a prognostic indicator in OSCC. The meta-analysis was executed at a random-effect model as a result of its low rate of heterogeneity (I^2 = 0%). Five eligible studies reported the prognostic value of CD163$^+$ TAM in OSCC. The pooled analysis revealed a high expression of CD163$^+$ TAM and overall survival (OS) corresponded to a worse survival in OSCC patients (HR = 2.64; 95% CI: [1.65, 4.23]; $p < 0.0001$) (**Figure 2**). Furthermore, in accordance to the stromal localisation of CD163$^+$ TAM, it revealed similar results with the association being significant in stromal expression in OSCC patients (HR = 3.56; 95% CI: [2.33, 5.44]; $p < 0.00001$) (**Figure 3**).

Tumour and Stromal CD68$^+$ TAMs Fail to Predict Prognosis in OSCC

Similar to findings of M2-like CD163$^+$ TAMs, the presence of CD68$^+$ TAMs is associated with poor prognosis in nasopharyngeal carcinoma (NPC), gastric and hepatocellular cancers, this study investigated whether CD68$^+$ TAMs could also be adopted as a prognostic indicator in OSCC. The meta-analysis was executed at a random-effect model as a result of its low rate of heterogeneity (I^2 = 41%). Four eligible studies reported the prognostic value of CD68$^+$ TAM in OSCC. It should be noted in this analysis, included studies evaluate the expression of CD68$^+$ TAM in more than one area (stroma and tumour (intra-tumoural) area). The pooled analysis revealed the association between high CD68$^+$ TAMs and OS showed no statistically significant difference in OSCC patients (HR = 1.26; 95% CI: [0.76, 2.07]; p = 0.37) (**Figure 4A**). In addition, CD68$^+$ TAM expression was evaluated in different sample locations (stroma vs. tumour). The subgroup analysis revealed no association between stromal (HR = 1.30; 95% CI: [0.55, 3.04]; p = 0.55) or tumour (intra-tumoural) (HR = 1.40; 95% CI: [0.40, 4.90]; p = 0.60) expression of CD68$^+$ TAMs and OS in OSCC patients (**Figure 4B**).

PD-L1 Expression May be Associated With a Positive Prognosis in OSCC

When considering the immune-suppressive nature of PD-L1, it was not surprising to note that this marker was associated with a poor prognosis in breast, bladder and non-small cell lung cancer as well as in malignant pleural mesothelioma and renal cell carcinoma, whereas the opposite prognosis was observed in breast cancer and HKSCC. The present study wished to investigate whether PD-L1 acted as either a positive or negative prognostic indicator. Five eligible studies reported the prognostic value of PD-L1 expression in OSCC. A high rate of heterogeneity was found (I^2 = 70%), therefore a random effect model was performed. In this analysis, one study evaluated PD-L1 expression twice (two areas of the sample from the same cohort) was included. Pooled analysis revealed the association of high PD-L1 expression and OS showed no statistically significant difference in OSCC patients (HR = 0.64; 95% CI: [0.35, 1.18]; p = 0.15) (**Figure 5A**). In addition, PD-L1 expression was evaluated in different sample locations (stroma vs. tumour). The subgroup analysis revealed no association between stromal (HR = 0.53; 95% CI: [0.23, 1.21]; p = 0.13) or tumour (intra-tumour) (HR = 2.24; 95% CI: [0.83, 6.02]; p = 0.11) expression of PD-L1 and OS in OSCC patients (**Figure 5B**).

DISCUSSION

This meta-analysis has reviewed the current literature on the prognostic potential of tissue biopsy tumour-associated macrophages; CD163$^+$ TAMs and CD68$^+$ TAMs as well as PD-L1 expression in OSCC. This meta-analysis demonstrated a significant association between high CD163$^+$ TAMs with poor survival/prognosis in OSCC patients. Additional results revealed

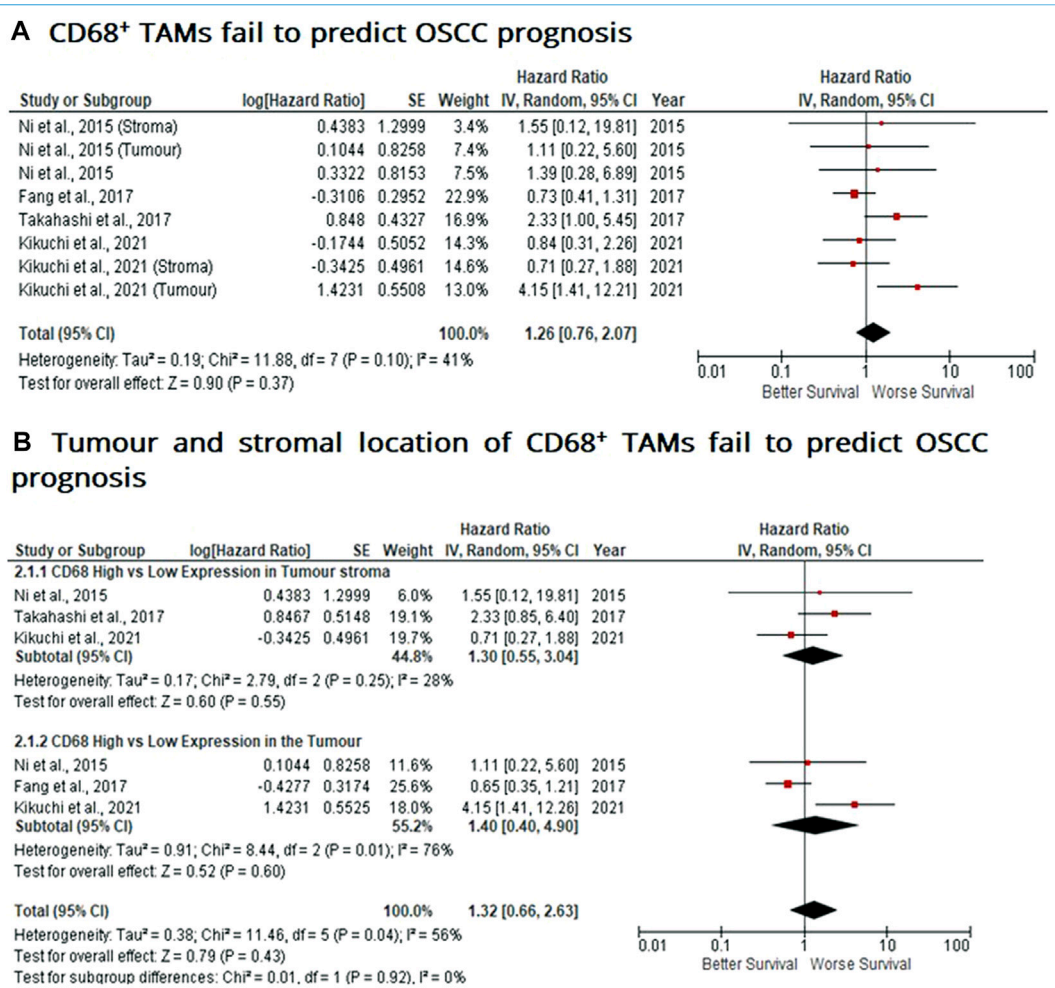

FIGURE 4 | Tumour and stromal CD68$^+$ TAMs fail to predict prognosis in OSCC Forest-plot reveals hazard ratios (HR) and 95% CI for the association of CD68$^+$ TAMs and OS in OSCC patients. **(A)** Studies were released evaluating the expression of CD68$^+$ TAMs in several areas of the samples from the same group, whereas **(B)** is a Forest-plot presenting hazard ratios (HRs) and 95% CI in a subgroup analysis related to survival in accordance to stromal or intra-tumour localisation of CD68$^+$ TAMs in OSCC samples. Red square represents hazard ratio for each study, horizontal lines represent 95% confidence intervals and vertical line represents line of no effect. Black diamond represents the mean weighted overall hazard ratio among all studies (pooled estimate). An HR >1 illustrates a higher risk of death or progression associated with high levels of CD68$^+$ TAMs. Tests for overall effect reveals statistical significance between CD68$^+$ TAMs and OS in OSCC patients (*p* value) according to tumour as a whole **(A)** or specific tumour location **(B)**.

an insignificant association of CD68$^+$ TAMs with OS, whereas PD-L1 expression approaches significance, indicating a potential positive prognosis associated with OSCC patients. Therefore, it reveals CD163$^+$ TAMs to exhibit the best prognostic potential of macrophage subsets in both intra-tumour and stromal OSCC biopsies.

The present pooled analysis revealed a high density of CD163$^+$ TAMs was associated with worse overall survival in OSCC (HR = 2.64; 95% Cl: [1.65, 4.23]; $p < 0.0001$). These findings are consistent in several other studies, reporting the correlation of CD163$^+$ macrophages and worse survival in breast, gastric, colorectal and hepatocellular cancers (24, 55–57). Also, pooled analysis found CD163$^+$ located within the tumour stroma to be associated with poor survival in OSCC patients (HR = 3.56; 95% Cl: [2.33, 5.44]; $p < 0.00001$). These findings were consistent with high CD163$^+$ TAM densities in the tumour stroma and associated with poor survival in SCCHN (squamous cell carcinoma of the head and neck) (26). Similarly, high levels of tumour stroma CD163$^+$ TAMs were associated with lymph node metastasis in OSCC (58). These high levels of OSCC CD163$^+$ TAMs could be explained by the ability of TAMs to directly stimulate EGF (epidermal-growth factor) as well as anti-inflammatory cytokines (such as IL-6, IL-10), pro-inflammatory cytokines such as TNF-α, and chemokines such as CXCL12, CCL16, CCL18. Collectively, these factors induce tumour cell growth and survival factors which enhance tumour cell proliferation, migration and metastasis (59, 60).

Interestingly, the expression of CD163 is not only restricted to TAMs but may also be associated with cell fusion where the fusion of cancer cells and TAMs can increase metastatic potential with migratory leukocytes in cancer patients and plays a role in cancer progression. This can lead to a more aggressive and

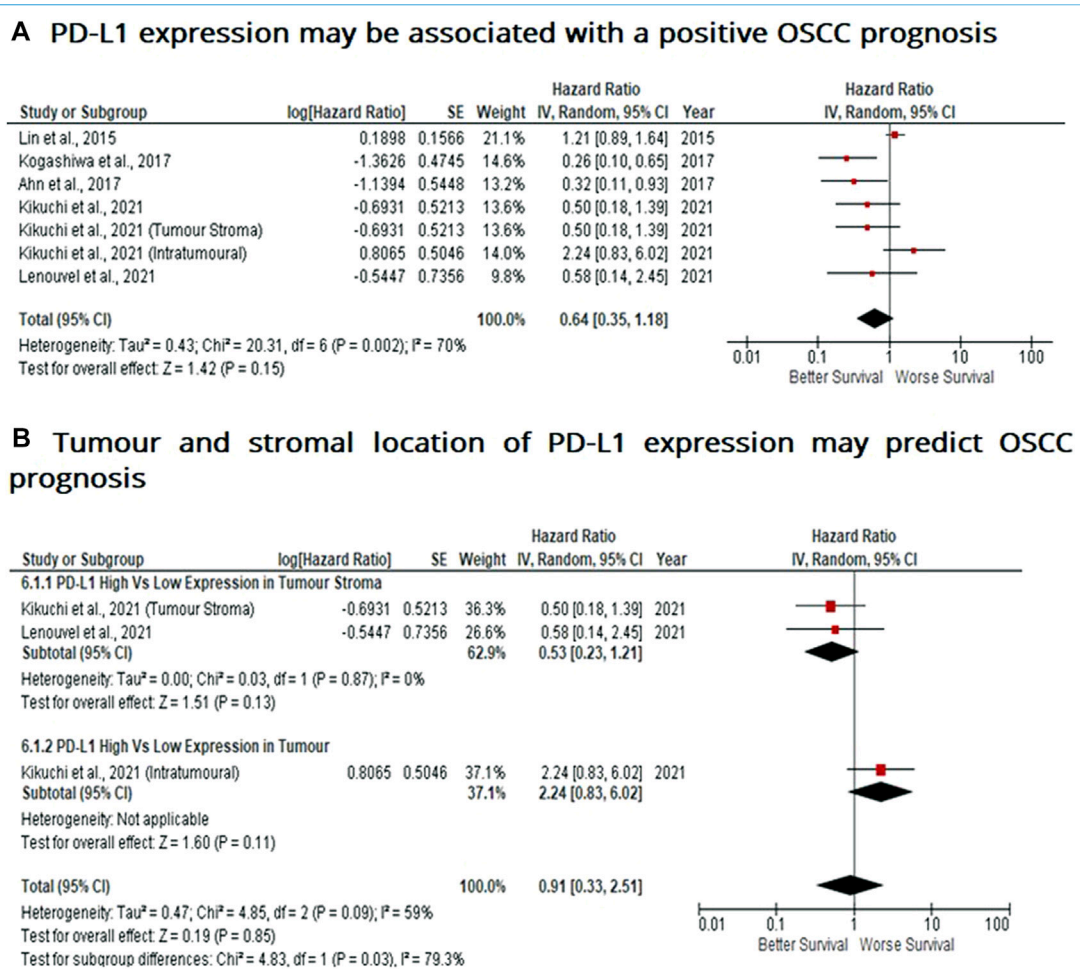

FIGURE 5 | PD-L1 expression may be associated with a positive prognosis in OSCC Forest-plot reveals hazard ratios (HRs) and 95% CI for the association of PD-L1 expression and OS in OSCC patients. **(A)** Studies evaluating the expression of PD-L1 in several areas of tumour samples from the same group, whereas **(B)** Forest-plot reveals hazard ratios (HRs) and 95% CI in a subgroup analysis related to survival in accordance to stromal or intra-tumour localisation of PD-L1 in OSCC samples. Red square represents hazard ratio for each study, horizontal lines represent 95% confidence intervals and vertical line represents line of no effect. Black diamond represents the mean weighted overall hazard ratio among all studies (pooled estimate). An HR <1 illustrates a better overall survival associated with PD-L1 expression. Tests for overall effect reveals statistical significance between PD-L1 expression and OS in OSCC patients (*p* value) according to tumour as a whole **(A)** or specific tumour location **(B)**. Percentage heterogeneity (I^2) is indicated and as such, results are conducted in a random-effect model.

metastatic phenotype causing an unfavourable prognosis as demonstrated in OSCC (61, 62). Therefore, it is imperative to discriminate $CD163^+$ malignant cells and macrophages when examining the influence of $CD163^+$ on prognosis. Nevertheless, many studies reveal consistent findings with the present results demonstrating CD163 may serve as a significant prognostic biomarker in OSCC. Interestingly, the results presented about $CD163^+$ TAMs may provide clinical implications. More specifically, they may serve as therapeutic targets for anticancer therapeutic regimens which may include the repolarisation of TAMs from M2-like TAMs to an M1-like phenotype, to restrain tumour progression (63). Therefore, a greater understanding of TAM function and OSCC progression is critical for future research in TAM-targeted therapies.

Compared to $CD163^+$ TAMs, pooled results demonstrated high $CD68^+$ TAMs were not statistically significant in revealing poor overall survival in OSCC patients (HR = 1.26; 95% Cl: [0.76, 2.07]; $p = 0.37$). However, other meta-analysis, has revealed high $CD68^+$ TAM densities were associated with worse overall survival and disease-free survival in nasopharyngeal carcinoma (NPC) patients (64). Similar studies revealed $CD68^+$ TAMs were associated with poor survival in gastric cancer and hepatocellular carcinoma, respectively (65, 66). These findings may be indicative of $CD68^+$ TAMs possessing immunosuppressive and pro-tumour responses, favouring cancer progression. Interestingly, $CD68^+$ TAMs have been shown to suppress cytotoxic activity of $CD8^+$ T-cells and increase tumour growth (67). On the other hand, in the case of oesophageal squamous cell carcinoma, $CD68^+$ TAMs were correlated with a favourable prognosis (68), suggestive that $CD68^+$ TAMs may also function as M1 macrophages, revealing pro-inflammatory and anti-tumour effects. Whilst

these molecular mechanisms are not fully understood, studies reveal TAMs may exert tumoricidal activity *in vitro;* more specifically, to polarise into M1 TAMs orchestrated by the production of IFN-γ which also activates cytotoxic $CD8^+$ T and NK cell responses to initiate tumour cell killing (12, 19).

Similar to findings in this study, $CD68^+$ TAMs did not significantly correlate with overall survival and recurrence-free survival (RFS) in multivariate analysis in basal-like breast cancer (BLBC) and triple-negative cancer of the breast (24, 69). In addition, no prognostic utility was found between $CD68^+$ TAMs and OS in SCCHN patients (26). These findings reveal $CD68^+$ TAMs may serve as a poor prognostic biomarker as demonstrated in this study focussed on OSCC, but more importantly, may indicate CD68 as a pan-macrophage marker expressed by both M1-like and M2-like TAMs, capable of exhibiting opposing effects on the tumour microenvironment (70). Therefore, this study's findings and the mounting conflicting evidence between different cancers, indicates that $CD68^+$ TAMs may be a poor prognostic biomarker in OSCC or at least requires further investigation across a variety of cancers/tumours as well as their TMEs.

Contrary to expectation, pooled results also revealed high PD-L1 expression had a non-significant positive impact on overall survival in OSCC patients (HR = 0.64; 95% Cl: [0.35, 1.18]; p = 0.15). A high level of heterogeneity (I^2 = 70%) among the included studies were revealed, demonstrating conflicting results with each other. In contrast to these data however, numerous studies reveal PD-L1 expression was associated with poor prognosis and overall survival (OS) in solid cancers, such as head and neck squamous cell carcinoma (HNSCC) (71), breast cancer (72), non-small cell lung cancer (NSCLC) (73) and bladder cancer (74). This association with poor prognosis may be suggested by PD-L1/PD-1 binding to suppress $CD8^+$ cytotoxic T-lymphocyte activation, leading to the evasion of the host immune anti-tumour response, thereby decreasing the survival rate in many cancers (37, 75). Additionally, whilst PD-L1 expression may protect macrophages from cell death, OSCC tumour cells induce TAM PD-L1 expression *via* IL-10 and induce T-cell apoptosis, further reinforcing an unfavourable prognosis (76).

Contradictory to these studies, yet consistent with findings in this investigation, PD-L1 expression in primary tumour cells was associated with prolonged DFS (Disease-free survival) in HNcSCC (head and neck cutaneous squamous cell carcinoma) (77). Similarly, high PD-L1 expression correlated better OS and DFS in breast cancer patients (78). This observation in prolonging survival in patients with PD-L1 expression may be due to the induction of an anti-tumour immune response. More specifically, IFN-γ, released by tumour-infiltrating lymphocytes as an adaptive immune-resistance mechanism to inhibit local effector T-cell function, can upregulate PD-L1 expression in tumour cells (36). Interestingly, IFN-γ also induces protein kinase D isoform 2 (PKD2), an important negative regulator of PD-L1 expression in OSCC. Thus PDK2 inhibits PD-L1 expression and promotes anti-tumour effects (blocking PD-1/PD-L1 dependent tumour antigen-specific $CD8^+$ T cell apoptosis) (79). In addition, a high expression of PD-L1 was not statistically associated with OS in oesophageal squamous cell carcinoma (80), and more recently, pooled analysis of high PD-L1 expression did not have a statistically significant association with OS, DFS, DSS (Disease-specific survival) in OSCC patients (81). The findings of this investigation (**Figure 5**) are consistent with these studies focussed on OSCC and oesophageal SCC. In addition, further subgroup analysis suggested that stromal expression of PD-L1 may be associated with improved survival, whereas intra-tumour PD-L1 expression may be associated with poor prognosis and overall survival. This may be indicative of $PD\text{-}L1^+$ cell location is predictive of survival and may reflect stage of cancer, or, when contrasted with the poor survival observed for $CD163^+$ TAMs, is suggestive that stromal PD-L1 and CD163 are expressed on different TAM subsets or that PD-L1 may not be expressed on TAMs at all. Thus, this current investigation goes some way to indicating PD-L1 as a prognostic marker of survival, or indeed stage of cancer progression in OSCC which may reach statistical significance with the inclusion of more clinical studies. Further investigation may also potentially validate PD-1/PD-L1 interaction as a future therapeutic target for OSCC.

CONCLUSION

In conclusion, this meta-analysis confirmed the prognostic role of $CD163^+$ TAMs, where a high cell number was associated with poor overall survival in OSCC. This indicates $CD163^+$ TAMs may be a useful novel prognostic biomarker for OSCC and may suggest TAMs as a potential therapeutic target. Both $CD68^+$ TAMs and PD-L1 revealed an insignificant correlation with overall survival in OSCC patients and limits the prognostic value of both biomarkers in OSCC, however the fact that the OS approached significance for PD-L1 is potentially indicative of PD-L1 being revealed as a positive prognostic indicator in the future.

SUMMARY TABLE

What is Known About Subject

- Presence of TAMs are associated with poor prognosis of tumours including OSCC.

What This Paper Adds

- In contrast to other cancers, $CD68^+$ TAMs fail to indicate OSCC prognosis.
- $CD163^+$ TAMs and expression of PD-L1 could serve as both prognostic indicators of survival and stage of tumour progression: counter-intuitive, as these markers are normally associated with M2 subset, which is described as pro-tumoral.
- TAM subset analysis and location (tumour or stroma) is indicative of OSCC stage and prognosis.

SUMMARY SENTENCE

- TAMs, and their location, are indeed, indicative of OSCC survival; where both tumour and stromal located $CD163^{+}$ TAMs are indicative of poor prognosis whereas stromal PD-L1 expression may be indicative of a better prognosis when compared to tumour expressed PD-L1.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MC conceived, designed experiments undertook experimental data capture, analysed data and drafted the manuscript. MP contributed to experimental design, data analysis and drafting of manuscript. PL-Y involved in experimental design, data interpretation and analysis, and edited manuscript. VS supervised the design of experimental approach, data interpretation and analysis, and edited this manuscript. AF supervised experimental design, data capture and analysis and edited this manuscript. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/bjbs.2023.11065/full#supplementary-material

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REVIEW
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CCAT 1- A Pivotal Oncogenic Long Non-Coding RNA in Colorectal Cancer

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Colorectal cancer (CRC) is ranked as the third most common cancer and second deadliest cancer in both men and women in the world. Currently, the cure rate and 5-year survival rate of CRC patients remain relatively low. Therefore, discovering a novel molecular biomarker that can be used to improve CRC screening, diagnosis, prognosis, and treatment would be beneficial. Long non-coding RNA colon cancer-associated transcript 1 (CCAT 1) has been found overexpressed in CRC and is associated with CRC tumorigenesis and treatment outcome. CCAT 1 has a high degree of specificity and sensitivity, it is readily detected in CRC tissues and is significantly overexpressed in both premalignant and malignant CRC tissues. Besides, CCAT 1 is associated with clinical manifestation and advanced features of CRC, such as lymph node metastasis, high tumor node metastasis stage, differentiation, invasion, and distant metastasis. In addition, they can upregulate oncogenic c-MYC and negatively modulate microRNAs *via* different mechanisms of action. Furthermore, dysregulated CCAT 1 also enhances the chemoresistance in CRC cells while downregulation of them reverses the malignant phenotypes of cancer cells. In brief, CCAT 1 serves as a potential screening, diagnostic and prognostic biomarker in CRC, it also serves as a potential therapeutic marker to treat CRC patients.

Keywords: biomarker, long non-coding RNA, colorectal cancer, CCAT 1, c-MYC

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INTRODUCTION

Colorectal cancer (CRC) is ranked as the third most common cancer and second deadliest cancer in both men and women in the world. In 2020, approximately 1,931,590 people are diagnosed with CRC and 935,173 people died because of CRC worldwide. In Malaysia, CRC is also the third-leading cancer and is recorded to have 6,597 new cases and cause 3,462 deaths in 2020 (1).

CRC is usually caused by a gradual buildup of gene mutations or sometimes by changes in epigenetic processes, which leads to abnormal activation of oncogenes and inactivation of tumor suppressor genes (2) It begins from benign adenomatous polyps to advanced adenoma with high-grade dysplasia or carcinoma *in situ*, invasive adenocarcinoma and ultimately, metastasizes to distant organs such as the liver (3).

Although the treatment of CRC has been improved, the cure rate and 5-year survival rate for CRC patients are still relatively low as many of them have already been diagnosed with stage III or IV CRC at their first visit (4). In addition, after potentially curative surgery or adjuvant therapies, one-third of the patients will still relapse (5). Therefore, early detection of benign colon lesions and recurrence of

disease through an effective screening method, such as using molecular biomarkers, is important to increase the chance of survival and significantly improve the overall outcome in CRC patients (6).

To date, a lot of evidence has revealed that long non-coding RNAs (lncRNAs) molecules are aberrantly expressed in CRC tissues or cells (7). LncRNAs are a class of regulatory non-coding RNAs (ncRNAs) that have the highest diversity, they are at least 200 nucleotides long and possess high tissue specificity as they are regulated by specific regulatory systems that are different from protein coding genes (8). As the name suggests, lncRNAs do not encode protein as they lack functional open reading frames (ORFs) (9). However, they can act as regulatory molecules to modulate cellular or biological processes such as cell proliferation, differentiation, and apoptosis through interacting with other cellular macromolecules like RNA, DNA, and proteins, and through regulating gene expression epigenetically, transcriptionally, and post-transcriptionally (10). LncRNAs are widely dysregulated in cancer, their expression level in cancer depends on whether they act as tumor driving genes or tumor suppressor genes, and dysregulated lncRNA triggers tumor carcinogenesis including CRC (11).

In 2012, Nissan et al. discovered a new oncogenic lncRNA that is aberrantly overexpressed in colon cancer using Representational Difference Analysis (RDA), cDNA cloning, and rapid amplification of cDNA ends (RACE) (12). This lncRNA is named colon cancer-associated transcript-1 (CCAT 1) or LOC100507056 and it is 2,628 nucleotides long (12). Since then, many studies also revealed that CCAT 1 is overexpressed in other human cancers, such as gastric cancer (13), lung cancer (14), breast cancer (15), ovarian cancer (16), gall bladder cancer (17), hepatocellular carcinoma (18), prostate cancer (19) and acute myeloid leukemia (20). Other than human cancers, CCAT 1 is also significantly expressed in inflammatory bowel diseases such as ulcerative colitis (UC) and Crohn's disease (CD) (21). In CRC, dysregulated CCAT 1 has been discovered to promote tumorigenesis by facilitating proliferation, metastasis, and anti-apoptosis of CRC cells through multiple mechanisms (12). Moreover, dysregulation of CCAT 1 also affects the developing chemoresistance in CRC cells (22). Therefore, it is suggested that CCAT 1 may be a potential molecular biomarker in screening, diagnosis, prognosis and act as a target for CRC treatment.

In this review, we describe the characteristics and identifications of CCAT 1, and its potential role in screening, diagnosis, prognosis and treatment of CRC. The mechanism of actions of CCAT 1 and the factors that cause CCAT 1 dysregulation in CRC are also elucidated. In the last, we describe the biological functions of CCAT 1 in CRC tumorigenesis, and how CCAT 1 contributes to the chemoresistance of CRC cells.

CCAT 1

CCAT 1 is an oncogenic lncRNA, its gene is located on human chromosome 8q24 (chr.8q24) region, specifically 8q24.21 nearby the *c-MYC* gene, which is one of the well-studied oncogenes (23). CCAT 1 RNA contains three short ORFs, which are nucleotides 95–208; 310–519 and 1,621–1,770 respectively. However, none of them can encode protein (12). In addition, CCAT 1 is multiexonic, it contains two exons which are nucleotides 1–288 and 289–2,612 respectively and is capped at the 5′ end and polyadenylated at the 3′ end (12, 24). Moreover, the promoter region of CCAT 1 contains an evolutionarily conserved enhancer box (E-box) (25). This E-box can be bound by c-MYC transcription factor which plays an extensive role in the initiation and development of most cancers (25). Furthermore, CCAT 1 consists of a short sequence at the 3′ end called microRNA response element (MRE) that complements the seed region or 5′ portion of certain micro-RNAs (miRNAs). It regulates the biological function of those miRNAs by targeting and interacting with them (26, 27) Taken together, all the characteristics stated above allow CCAT 1 to play roles in CRC tumorigenesis.

Isoforms: CCAT1-S and CCAT1-L

According to the GENBANK nucleotide sequence database, CCAT 1 produces two isoforms: short isoform CCAT1-S and long isoform CCAT1-L (28). CCAT1-S is 2,628 nucleotides long, it was identified in colon cancer in 2012; whereas CCAT1-L is 5,200 nucleotides long, it was discovered in CRC in 2014 (12, 24). The short isoform CCAT1-S is also referred as CCAT 1 or cancer-associated region long noncoding RNA-5 (CARLo-5), it is named as CCAT1-S after the long isoform CCAT1-L is discovered (12). Same as CCAT1-S, CCAT1-L is also transcribed from 8q24, 515 kb upstream of *c-MYC* gene (MYC-515), a tumor type-specific super enhancer region of *c-MYC* with a length of 150 kb (24). Therefore, CCAT1-L may also be identified as an enhancer-derived RNA (eRNAs) for *c-MYC* (24). In addition, CCAT1-L also contains two exons, and it is 3′-capped and 5′-polyadenylated although eRNAs are not spliced or polyadenylated in general (28).

The relationship between CCAT1-S and CCAT1-L and their characteristics were further studied by Xiang et al. They found out that there is a spatial structure overlap between CCAT1-L and CCAT1-S in which two exons of CCAT1-L overlapped with CCAT1-S, and reduced CCAT1-L causes an immediate disruption of CCAT1-S (24) Therefore, CCAT1-S is suggested to be derived from CCAT1-L and there may be a correlation between them. In addition, both are localized in different subcellular compartments. After transcription, CCAT1-S is transferred to the cytoplasm whereas CCAT1-L is retained in the nucleus, more accurately, near or at its site of transcription (24). Moreover, CCAT1-S is highly expressed in CRC (12), gastric cancer (13), gallbladder cancer (17), and hepatocellular cancer (18) whereas CCAT1-L is only overexpressed in CRC (24). Recent evidence reveals that CCAT1-L is also overexpressed in gastric adenocarcinoma and hepatocellular carcinoma, hence CCAT1-L is no longer specifically expressed only in CRC (29, 30).

Potential Roles of CCAT 1 in CRC

CCAT 1 is found significantly overexpressed in both early and late stages of CRC patients (31, 32). There was a significant differential expression of CCAT 1 in CRC tumour tissues and

normal adjacent tissues in which CCAT 1 is upregulated in CRC tumour tissues as compared to normal colon mucosa (32). Current studies in Singapore showed that the expression of CCAT 1 in patients' tumours was hundreds of times higher as compared to their matched normal mucosa (33). Besides, high expression of CCAT 1 can be detected in every stage of the mucosal adenoma-carcinoma sequence in CRC, either pre-malignant or malignant tissues, such as benign adenomatous polyps, primary colon adenocarcinoma including lymph nodes and liver metastases (31). In addition to colorectal tumor tissues, CCAT 1 is also highly expressed in peripheral blood mononuclear cells (PBMC) of colon cancer patients (12). Furthermore, the high expression level of CCAT 1 can also be detected in colon cancer-associated lymph nodes. Siddique et al. also revealed that the plasma CCAT 1 expression in CRC patients exceeds 4.54-fold than in normal individuals (34). Not only in tissue and plasma samples, the expression of CCAT 1 also showed significant differences in stool samples from CRC patients and healthy individuals, with the former being 4.5 times higher than the latter (35). The extremely high ratio of tumour to normal tissue highlights that CCAT 1 is specific and these findings suggest that CCAT 1 may be used as a screening and diagnostic biomarker for CRC tissues. Intriguingly, Zhao et al. revealed that using CCAT 1 together with another lncRNA HOTAIR can improve CRC screening and detect CRC at an early stage as possible. The authors agree with the high diagnostic power of plasma CCAT 1, with the indication of 85.3% specificity and 75.7% sensitivity (36). Besides, Kam et al. demonstrate that thiazole orange-peptide nucleic acid molecular beacon (TO-PNA-MB) complementary to CCAT 1 detects CRC in human biopsies based on the FISH method and the results showed satisfactory results in which higher fluorescence intensity was seen in benign adenoma and adenocarcinoma tissues as compared to their matched normal colonic tissues (37).

Secondly, although the utility of prognostic biomarkers in a clinical setting is less than screening and diagnostic biomarkers, they are nevertheless useful in evaluating a patient's likely outcome regardless of treatment (38). A meta-analysis demonstrated that CCAT 1 expression affects CRC patients' clinical stage and their overall survival (OS) (39). Patients that have high CCAT 1 expressed in CRC tissues have shorter survival times and poorer disease-free survival (40). In addition, increased CCAT 1 is also significantly correlated with advanced clinical features of CRC, such as lymph node metastasis (LNM), high tumor node metastasis (TNM) stage, differentiation, microvascular invasion, and distant metastasis (41). In short, CCAT 1 can be utilized as a potential prognostic biomarker to evaluate patients' clinical outcomes and predict their survival rate regardless of the metastasis stage and treatment in CRC.

Strikingly, CCAT 1 can also be used to predict the therapeutic effects of CRC patients so that a suitable and effective treatment can be proposed. Specific targeted treatment can be restricted to CRC patients expressing CCAT 1 (38). JQ-1 treatment is a targeted therapy that uses bromodomain and extra-terminal (BET) protein inhibitors to target BET proteins (42). During the development of CRC, BET protein accumulates and binds at super-enhancers of *c-MYC*, thereby activating the transcription of *c-MYC* gene in a tumor type-specific and lineage-dependent manner (43). Since CCAT 1 is situated at 500 kb upstream of *c-MYC* promoter, which is the super-enhancer region of *c-MYC*, it may be bound by this BET protein family (43). CCAT 1 is significantly downregulated in CpG island methylator phenotype positive (CIMP+) colon cancer cells upon JQ1 treatment, this indicates that CCAT 1 is sensitive to BET inhibitors and can be directly regulated by BET protein (43). Since CCAT 1 is sensitive to BET inhibition and its expression predicts JQ1 sensitivity as well as BET-mediated *c-MYC* regulation, it can serve as a potential biomarker in which its expression level can help identify patients who can well-respond and are most likely to benefit from BET inhibitor treatment (43). Undeniably, this patient selection strategy will be very useful in clinical trials. However, as super enhancers cannot be detected consistently in human tissues, CCAT 1 cannot be used as a predictive biomarker for companion diagnostic assay (43).

In addition to screening, diagnosis and prognosis, CCAT 1 may serve as a target for onco-lncRNA targeted therapy, which could be a promising treatment option for CRC patients (44). Study shows that the expression of CCAT 1 can be significantly reduced by siRNA. Downregulation of CCAT 1 upregulates the expression of cyclin dependent kinase inhibitor 1A (CDKN1A) mRNA, which regulates G1 cell cycle arrest and leads to a reduction in colon cancer cell proliferation (14, 45) Moreover, by knocking down CCAT 1, the malignant characteristic of CRC cells, such as migration and invasion can be reversed (28). For instance, the authors discovered that Ginsenoside Rg3, an anti-cancer compound, can downregulate CCAT 1 thereby inactivating the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway and eventually inhibiting proliferation, migration and invasion of CRC cells (46). Furthermore, downregulation of CCAT 1 also induces CRC cell apoptosis by increasing proapoptotic protein Bcl-2-associated X protein (BAX) expression levels *via* p53 signaling pathway (47).

In brief, CCAT 1 may serve as a useful biomarker in screening, diagnosis and prognosis of CRC; it may also serve as a target for onco-lncRNA targeted therapy for CRC patients and facilitate in selecting patients that can respond well to a specific treatment. However, targeting or using CCAT 1 as a treatment approach requires a more comprehensive knowledge of its mechanisms of action and its biological functions in CRC, and they are described below.

MECHANISMS OF ACTION OF CCAT 1 IN CRC

lncRNAs are important gene expression regulators. They function as protein scaffolds, transcription coactivators or inhibitors, and mRNA decoys or microRNA sponges to regulate their expression thereby modulating biological or cellular processes (48). Considering that CCAT 1 is a lncRNA, it should also exhibit some of these functions. Until now, there are four mechanisms of action of CCAT 1 found in cancer, however, only two mechanisms of action of CCAT 1 are known in CRC (23). As mentioned, CCAT1-L and

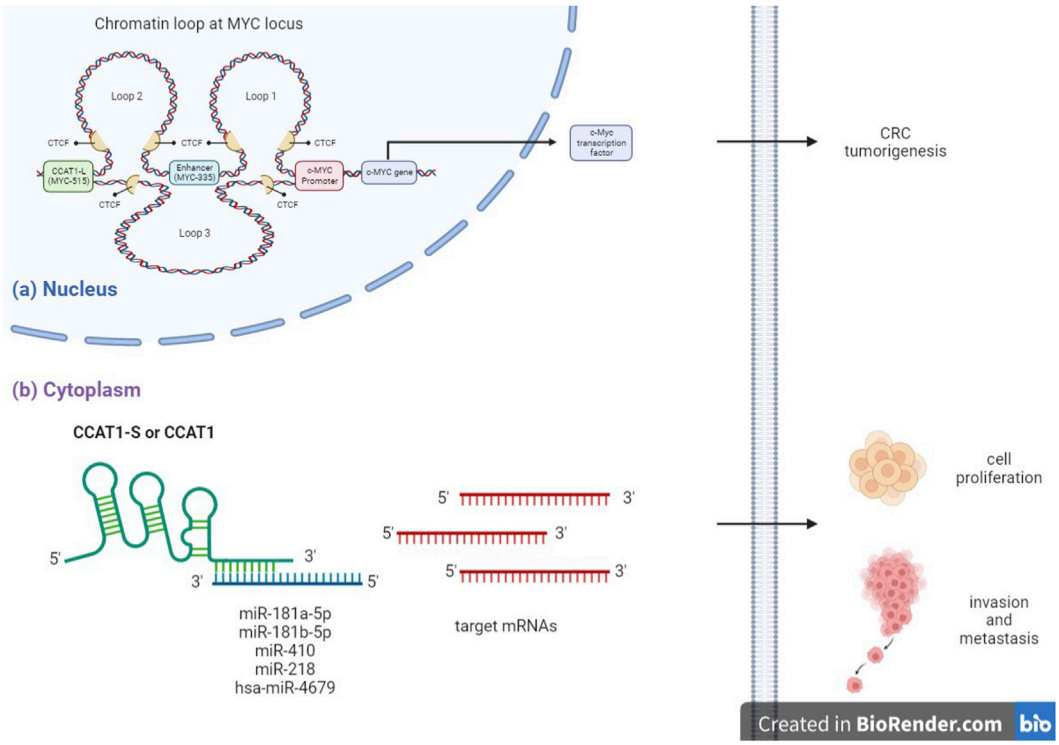

FIGURE 1 | Mechanism of action of CCAT 1 in CRC. **(A)** Nuclear lncRNA CCAT1-L, transcribed from distal tumor type-specific super-enhancer of c-MYC (MYC-515), interacts with CCCTC-binding transcription factor (CTCF) subsequently mediating intra-chromosomal interaction of well-studied oncoproteins c-MYC, which plays important role in CRC tumorigenesis. **(B)** Cytoplasmic lncRNA CCAT1-S or CCAT1 directly interacts with tumor suppressive miRNAs subsequently regulating miRNA target mRNAs translation and eventually promoting colon cancer cell proliferation, invasion and metastasis.

CCAT1-S are localized in different subcellular compartments, and this leads them to exert different regulatory roles at their particular sites of action.

Firstly, nuclear lncRNA CCAT1-L acts as an oncogene by binding to CCCTC-binding transcription factor (CTCF), regulating the intra-chromosomal interaction of well-studied oncoproteins c-MYC causing CRC tumorigenesis. Whereas cytoplasmic lncRNA CCAT1-S or CCAT 1 act as an oncogene by directly interacting with tumor suppressive miRNAs thereby upregulating miRNA target mRNAs translation and eventually promoting CRC proliferation, invasion and metastasis (**Figure 1**) (24, 49).

CCAT1-L Forms Long Range Chromatin Loop With the c-MYC Oncogene at the 8q24 Locus

Remarkably, there are several loci commonly found mutated in cancer, including the 8q24 locus (50). Within chromosome 8q24, there is a segment termed "gene desert" with approximately 3Mb long (51). This segment encompasses single nucleotide polymorphisms (SNPs) that cause increased susceptibility to various cancers, such as CRC, prostate cancer, breast cancer, esophagus cancer, ovarian cancer and pancreas cancer (52). Intriguingly, *c-MYC* gene is located just a few hundred kilobases telomeric to those mutational hot spots within this chromosome. It plays a vital role in maintaining CRC cell identity and promoting oncogenic transcription (51, 53). *c-MYC* upstream regulatory elements such as enhancers and super-enhancers that are located at MYC-515 regulate the transcription of *c-MYC* gene in a tissue or tumor type-specific manner (54). This super-enhancer involves maintaining the stability of chromatin looping at *MYC* locus through the formation of two chromatin loops with *c-MYC* promoter and a transcriptional enhancer for *c-MYC* gene (MYC-335) (24).

Given that protein coding genes are rarely found in gene desert near *c-MYC*, coupled with the emergence of lncRNAs, many researchers have attempted to discover lncRNAs that may regulate c-MYC to promote CRC tumorigenesis (54). To date, multiple lncRNAs that regulate *c-MYC* gene expressions such as CCAT 1, CCAT1-L and Colon Cancer Associated Transcript 2 (CCAT2) have been discovered (55). Interestingly, studies have shown that c-MYC expression in the tumour was significantly correlated to CCAT 1 (33). As mentioned before, CCAT1-L is transcribed from MYC-515, therefore, this allows it to play a role in regulating local gene expression and organizing chromatin structure (24). Furthermore, CCAT1-L is observed accumulating in-cis at or near its site of transcription in the nucleus (24). It can act as a cis-regulatory element to transcriptionally activate *c-MYC* by binding to it. Taken together, CCAT1-L serves as an enhancer-derived RNA (eRNA) and chromatin regulator for

c-MYC by upregulating *c-MYC* transcription and promoting long range chromosomal interactions at *MYC* locus (28).

Mechanism of Action of c-MYC

c-MYC is a tumor driving gene that was found overexpressed in numerous cancers including CRC. It is located at ~335kb telomeric of rs6983267 on chromosome 8q24, single nucleotide polymorphisms (SNPs) that were found to be associated with CRC (56, 57). Its expression is regulated by *c-MYC* proto-oncogene, which gives an immediate response after the activation of numerous ligand-membrane receptor complexes as its location is at the intersections of many growth-promoting signal transduction pathways (54, 56). *c-MYC* oncogene is a downstream regulatory gene of the PI3K/Akt signaling pathway, which is one of the most important pathways in CRC, and its activation reduces cell apoptosis and promotes cell proliferation (58, 59). *c-MYC* oncogene encodes an oncoprotein called c-MYC, which is a general transcription factor that regulates the expression of the gene that changes the characteristics of epithelial stem cells of colon tissues (60). As a typical transcription factor, c-MYC oncoprotein binds to promoters of genes or recruits histone acetyltransferases (HATs) epigenetically to regulate gene expression (54). c-MYC regulates 15% of all genes by binding to the enhancer box (E-box) with the sequence of 5′-CACGTG-3′ which is present in the promoter region of those genes, and this includes CCAT 1 (61). To function, c-MYC dimerizes with a protein called Max to form a transcriptional competent factor complex. This complex then binds target DNA sequences or E-boxes of target genes which are involved in cell proliferation and growth, differentiation, apoptosis and adhesion such as *cyclin-dependent kinase 4 (CDK 4)* (55). As such, c-MYC augments their expression thereby promoting cell proliferation and growth.

Under normal conditions, *c-MYC* gene expression is strictly controlled by many transcriptional regulatory motifs that are found within its promoter region with different mechanisms of action (54). When chromosomal translocations and aberrant signal transduction occur, *c-MYC* transcription is dysregulated, resulting in sustained *c-MYC* expression and an increased level of c-MYC transcription factor (62). Then, the increased c-MYC binds to E-boxes of target genes to command them, enabling the cells to grow and divide persistently, thus initiating the neoplasia formation (54).

How CCAT 1-L Regulates *c-MYC* Transcription

So how does CCAT1-L regulate *c-MYC* transcription when it needs to across 515 kilobases, which is such a large genomic distance? The answer will be the formation of long-range chromatin loops, which have been recognized to bring genes side by side to the enhancers (63). For that reason, CCAT1-L positively regulates the expression of *MYC* transcription by forming and promoting long-range chromatin interactions between *MYC* and its upstream regulatory elements (24). Xiang et al. demonstrated that CCAT1-L participates in the first two of the three chromatin loops generated at *MYC* locus: loop 1 connects *c-MYC* promoter to MYC-335; loop 2 connects MYC-335 to MYC-515, loop 3 connects MYC-515 to *c-MYC* promoter (24). CCAT1-L is shown to play a role in maintaining the stability of enhancer-promoter looping at *MYC* locus in CRC cancer cells by recruiting a chromatin loop forming factor called CCCTC-binding factor (CTCF) (23). When CCAT1-L interacts with CTCF, it promotes the chromatin interactions between the *c-MYC* promoter and its upstream enhancers, thereby activating the transcription of *c-MYC*. Moreover, studies reveal that in-cis accumulation of this lncRNA further promotes *c-MYC* transcription and enhances CRC tumorigenesis (24). In contrast, knockdown of CCAT1-L decreases *c-MYC* expression. When CCAT1-L is knocked down, it reduces the chromatin interactions between *c-MYC* promoter and its enhancers, therefore, *c-MYC* transcription will be reduced (24). Then, reduced expression of *c-MYC* leads to reduced translation of c-MYC, which eventually causes a reduction in CRC cell proliferation.

CCAT 1 Functions as a Molecular Sponge for miRNAs

A growing number of reports suggest that both lncRNAs and miRNAs have participated in CRC development and progression through lncRNA-miRNA-mRNA cross talk. They act as competing endogenous RNAs (ceRNAs) to regulate CRC cell proliferation, differentiation and apoptosis (64,65). miRNAs are small and highly conserved non-coding RNAs with a length of 18–24 nucleotides that regulate the translation and stability of specific target mRNAs (66). They can either be tumor suppressor genes or oncogenic miRNA, depending on the microenvironment in the cells that they are expressed (66). Recent studies suggest that the relationship between CCAT 1 and miRNAs in CRC is double negative feedback or reciprocal repression (18). Cytoplasmic lncRNA CCAT 1 functions as a molecular sponge or decoy for miRNAs. It changes the biological function of miRNA at the transcriptional level, thereby changing the expression of miRNA target genes indirectly (18). CCAT 1 contains a binding site for miRNAs which is the miRNA response element (MRE) at the 3′ end. It acts as a miRNA sponge that bind to certain miRNAs to inhibit their endogenous suppressive or oncogenic effects on their targets (49). As a result, CCAT 1 indirectly increases the expression of miRNA target genes (18).

Remarkably in CRC, several miRNAs are found to be the potential targets of CCAT 1, such as miR-124, miR-490-3p, miR-194, miR-24 and miR-181a-5p (47). However, only miR-181a-5p has inversely correlated with CCAT 1 expression (47). Subsequently, other studies reveal that miR-181b-5p, miR-218, miR-410 and hsa-miR-4679 are also the functional targets of CCAT 1. By binding and sponging these miRNAs, CCAT 1 induces the proliferation, invasion, and metastasis of CRC cells. It also inhibits cell cycle arrest and apoptosis of CRC cells (**Table 1**) (47, 49, 67–69).

FACTORS INVOLVED IN DYSREGULATION OF CCAT 1 IN CRC

Although many studies have revealed that CCAT 1 is associated with CRC tumorigenesis, the underlying mechanism that causes

TABLE 1 | miRNAs in CRC that are functional targets of CCAT 1

miRNA	Types of miRNA	Expression in CRC	Experimentally validated microRNA targets	Functions	Ref.
miR-181a-5p	Tumor suppressor	Low	P53	Suppress cell proliferation, mobility and invasion, and promote cell apoptosis	(47)
miR-181b-5p	Tumor suppressor	Low	Tumor suppressor candidate 3 (TUSC3)	Suppress cell proliferation, migration and invasion	(49)
miR-218	Tumor suppressor	Low	Vascular endothelial growth factor (VEGF)	Inhibit cell viability, promote apoptosis and reduce VEGF expression	(67)
miR-410	Tumor suppressor	Low	Inositol-Trisphosphate 3-Kinase B (ITPKB)	Suppress cell proliferation, migration and invasion, promote apoptosis	(68)
hsa-miR-4679	Tumor suppressor	Low	Guanine nucleotide-binding protein, gamma 10 (GNG10)	Suppress cell proliferation, migration and invasion, promote apoptosis	(69)

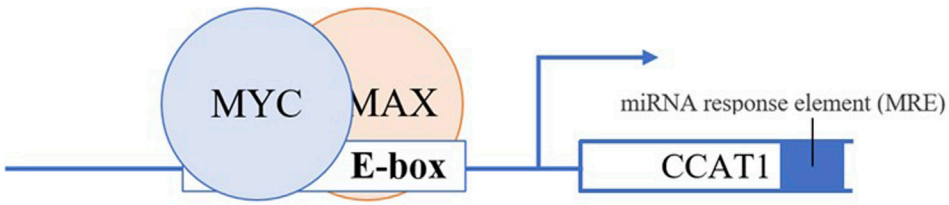

FIGURE 2 | c-Myc dimerizes with a protein called Max to form a transcriptional competent factor complex, this complex then binds the enhancer box (E-box) of CCAT1 at the promoter region thereby augmenting the expression of CCAT1.

CCAT 1 dysregulation in CRC has not been dealt with in depth. Many lines of evidence suggest that aberrant expression of lncRNAs can be caused by genetic alterations as well as epigenetic regulation. Indeed, SNPs, copy number alterations, or mutations within the non-coding genome alter the transcription of lncRNA (10, 70). Several SNPs are related to the expression of cancer-associated lncRNAs including CCAT2 and Prostate cancer-associated transcript 1 (PCAT-1) in CRC (71, 72). In addition, SNPs in lncRNA promoter region also modulate the expression and function of the lncRNA. Intriguingly, Li et al. found that the presence of a SNP rs67085638 in the 3′ UTR of CCAT 1 increases the expression of CCAT 1 (73). Other than SNPs, genomic rearrangements such as deletions, amplifications, or translocations within lncRNA loci may also alter its expression (74). However, there are currently no studies focusing on this aspect.

Dysregulation of CCAT 1 also mediated by transcriptional regulation of key transcription factor c-MYC (44). CCAT 1 and c-MYC seem to form a double-positive feedback loop to enhance the expression of each other. Growing evidence indicates that the transcription of CCAT 1 can be activated and upregulated by c-MYC (44). Overexpression of c-MYC reciprocally augments the expression of CCAT 1 by binding to the E-box in the promoter region of CCAT 1, consequently accelerating the development and metastasis progress of CRC, and *vice versa* (**Figure 2**) (44). Interestingly, oncogenic SNP rs6983267 within the *c-MYC* enhancer region increase CCAT 1 expression by long-range interaction with CCAT 1 promoter region (45). In addition, studies suggest that overexpression of CAMP responsive element binding protein 1 (CREB1), a phosphorylation-dependent transcription factor cause upregulation of CCAT1 (75).

Besides genetic alterations, epigenetic regulation such as DNA methylation, gene imprinting, chromatin remodeling, histone modification as well as non-coding RNAs regulation cause lncRNAs dysregulation (76). Studies demonstrate that the chromatin state of lncRNAs has been modified during diseases and this affects the expression of lncRNAs (77). For instance, the transcription of lncRNA maternally expressed gene 3 (MEG3) is repressed notably in hepatocellular cancer due to hypermethylation in its promoter region (77).

BIOLOGICAL FUNCTIONS OF CCAT 1 IN CRC

Many studies reveal that CCAT 1 promotes tumorigenesis in CRC by different mechanisms of action. As stated above, knockdown of CCAT 1 reduces colon cancer cell proliferation, reverses CRC cell invasion and metastasis, and improves CRC cell apoptosis (28, 45, 47). All these results indicate that CCAT 1 does exhibit oncogenic activities in CRC.

Thus, how CCAT 1 facilitates tumorigenesis in CRC? Current studies suggest that CCAT 1 induces CRC cell proliferation through upregulating oncoproteins c-MYC and oncogenic mRNA tumor suppressor candidate 3 (TUSC3), the target of miR-181b-5p in CRC cells (24, 49); enhances glucose metabolism to provide energy supply for the growth of colon cancer cells (78); facilitates CRC cell migration and invasion through accelerating EMT process and negatively modulate miR-218 as well as hsa-

TABLE 2 | Biological functions of CCAT1 in CRC.

Function	CCAT1 promotes CRC cell proliferation	CCAT1 facilitates CRC cell migration and invasion	CCAT1 inhibits CRC cell apoptosis
Mechanism of action	CCAT1 upregulates glycolytic pathway in colon cancer cells CCAT1 sponges miR-181b-5p thereby upregulating Tumor Suppressor Candidate 3 (TUSC3) CCAT1 upregulates c-MYC transcription factor	CCAT1 sponges hsa-miR-4679 thereby upregulating GNG10 CCAT1 sponges miR-218 thereby upregulating vascular endothelial growth factor (VEGF) CCAT1 accelerates EMT process	CCAT1 sponges miR-181a-5p thereby downregulating proapoptotic protein BAX

miR-4679 (67, 69), and lastly inhibits colon cancer cell apoptosis by sponging miR-181a-5p (**Table 2**) (47).

CCAT 1 Promotes CRC Cell Proliferation

CCAT1-L promotes CRC cell proliferation *via* upregulation of c-MYC transcription factor, which has been discussed previously (24). Furthermore, CCAT 1 also promotes CRC cell proliferation through an axis called CCAT1/miR-181b-5p/TUSC3 (49). By sponging miR-181b-5p in CRC cells, CCAT 1 positively regulates the expression of TUSC3 which in turn promote proliferation, migration, invasion, and accelerates tumor growth (49). CCAT 1 upregulates the glycolytic pathway in colon cancer cells by increasing the expression levels of glycolysis rate-limiting enzymes in colon cancer cells and promoting lactic acid production (78). This action provides energy supply for the proliferation of colon cancer cells as malignant tumors primarily rely on the glycolytic pathway for energy supply (78). Cui et al. also show that high glucose levels or hyperglycemia enhance the oncogenic effect of CCAT 1 on colon cancer cell proliferation, anti-apoptotic and migration (78).

CCAT 1 Facilitates CRC Cell Migration and Invasion

Evidence reveals that CCAT 1 facilitates the migration and invasion of colon cancer cells by accelerating epithelial-mesenchymal transition (EMT) process, in which the tight junctions between epithelial cells undergo dissolution, the polarity between apical and basal domains of epithelial cells are disrupted, and the cytoskeletal reorganized abnormally (79, 80). EMT process enables cancer cells to achieve invasive and migrative abilities so that they can isolate from primary tumor to invade and metastasize to distant organs such as the liver (79, 80). In CRC, CCAT 1 expression is associated with the expression of EMT markers, which are N-cadherin and E-cadherin (79). N-cadherin is a mesenchymal marker of the EMT while E-cadherin is an epithelial marker expressed in most normal epithelial tissues (81). When CCAT 1 is overexpressed, the expression of E-cadherin is downregulated whereas the expression of critical indicators of EMT including N-cadherin and vimentin is upregulated (82). This suggests that CCAT 1 may mediate CRC cell migration and invasion by accelerating EMT process.

Besides, overexpression of CCAT 1 can increase vascular endothelial growth factor (VEGF) expression by sponging miR-218, leading to an increase in CRC cell viability, proliferation, migration and invasion (67). MiR-218 has been suggested to inhibit the expression of VEGF that promotes angiogenesis, which is essential for cancer development and growth (67, 83). The study demonstrates that CCAT 1 and miR-218 have complementary binding sites. CCAT 1 can directly bind to miR-218 to inhibit its suppressive role on VEGF, thereby promoting CRC cell migration, invasion and viability (67).

A recent study demonstrates that CCAT 1 promotes progression of CRC *via* interaction between hsa-miR-4679 and GNG10 (69). GNG10 is a subunit of G-protein and was previously shown to have a potential role in melanoma tumorigenesis (84). In CRC, GNG10 was highly expressed whereas hsa-miR-4679 has low expression (69). By sponging tumor suppressor hsa-miR-4679, CCAT 1 upregulates GNG10 expression leading to CRC cell migration and invasion.

CCAT 1 Inhibits CRC Cell Apoptosis

CCAT 1 acts as competing endogenous RNAs (ceRNAs) to regulate tumor suppressors and apoptosis signaling pathways in CRC. For instance, CCAT 1 serves as a miRNA sponge for tumor suppressive miR-181a-5p that regulates the expression of apoptosis-related proteins BAX and B-cell lymphoma-2 (BCL-2) which are involved in the p53 signaling pathway and subsequently affect the proliferation of CRC cells (47). As CCAT 1 contains MRE that captures miR-181a-5p, it plays a tumor promoter role by binding to miR-181a-5p and abating the effect of miR-181a-5p on its own target pro-apoptotic protein BAX in CRC cells (47). As such, the expression level of BAX proteins reduces, leading to a reduction in CRC cell apoptosis (47). In opposite, the downregulation of CCAT 1 or upregulation of miR-181a-5p increases the expression levels of BAX *via* the p53 signaling pathway, resulting in accelerated CRC cell apoptosis (47).

ROLES OF CCAT 1 IN RESISTANCE TO CHEMOTHERAPY

Besides surgery, traditional chemotherapy drugs and advanced molecular target therapy are important means to destroy cancer cells, they can be used to manage patients with primary and metastatic CRC (85). However, the treatment of CRC has been challenging because it is a molecularly heterogeneous disease in which the tumors harbor distinct molecular features with different levels of sensitivity to treatments (86). Moreover, all malignant colon

cells manifest chemotherapy-related resistance (87). Therefore, in some cases, chemotherapy alone can hardly provide a complete cure, and this brings up a critical problem (87).

5-fluorouracil (5-FU) is the first-line chemotherapy for CRC patients. It is a synthetic fluorinated pyrimidine analogue that can interfere with DNA synthesis by irreversibly inhibiting the action of thymidylate synthase or incorporating its metabolites into DNA, thus leading to DNA damage and cell death (88). However, it is found that CRC patients often develop resistance to 5-FU-based chemotherapies, and this leads to a poor prognosis for patients. A recent study shows that nearly half of the patients that have metastatic CRC are resistant to 5-FU-based chemotherapies, therefore, finding out the resistance mechanisms is of upmost important (22). Chun Yang et al. demonstrate that downregulation of CCAT 1 significantly reverses the drug resistance of 5-FU-resistant colonic neoplasm cell lines by accelerating cell apoptosis (22). Although the underlying mechanism remains unclear, it provides a new direction for colon cancer treatment.

Another strong chemotherapy drug used to treat CRC patients is paclitaxel (PTX) (89). PTX exerts anti-tumor functions by inhibiting CRC cell proliferation through cell cycle arrest and preventing angiogenic features of endothelial cells (89). Fascin Actin-Bundling Protein 1 (FSCN1), which is the functional target of miR-24-3p, plays a key role in miR-24-3p-mediated sensitivity to paclitaxel (90). FSCN1 is an actin binding protein, it promotes cell migration, adhesion, invasion through EMT process, and its expression reduces the chemosensitivity of cancer cells to paclitaxel (91). CCAT 1 enhances chemoresistance of CRC cancer cells to PTX by regulating the expression of miR-24-3p as well as the expression of FSCN1 (90). CCAT 1 negatively modulates the expression of miR-24-3p to elevate the expression of FSCN 1 mRNA, leading to increased chemoresistance of CRC cells to PTX. Interestingly, the presence of the SNP rs67085638 in the CCAT 1 3′ UTR region increases the expression of CCAT 1 and enhances the chemoresistance to PTX in CRC cells (90). Nonetheless, downregulation of CCAT 1 can significantly restore the sensitivity of colon cancer cells to PTX (90). This highlights that CCAT 1 may be the hope in future therapeutic approaches in CRC.

CONCLUSION

CCAT 1 is a pivotal oncogenic lncRNA that may serve as a potential biomarker in the screening, diagnosis, prognosis, and treatment of CRC. All in all, the development of reliable diagnostic assays and effective therapeutic methods will be facilitated by a better knowledge of the roles of CCAT1 in CRC including its interaction with miRNAs, and this could significantly improve the long-term survival rate of CRC patients and reduce CRC morbidity and mortality. However, even though several studies have looked at the mechanisms of action of CCAT 1 in CRC, the factors that cause dysregulation of CCAT 1 in CRC are still not well understood. Moreover, there has been very little discussion about the underlying mechanism that causes CCAT 1 dysregulation in CRC, particularly epigenetic regulation. Other than that, many functional targets still have not been proven to correlate with CCAT 1 expression in colorectal cancer. Therefore it is likely to be some time before CCAT 1 can be clinically used as a biomarker in CRC. Further exploration is required to allow gaps in our current knowledge to be filled and for research in this area to progress further.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SPHINX-Based Combination Therapy as a Potential Novel Treatment Strategy for Acute Myeloid Leukaemia

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Introduction: Dysregulated alternative splicing is a prominent feature of cancer. The inhibition and knockdown of the SR splice factor kinase SRPK1 reduces tumour growth *in vivo*. As a result several SPRK1 inhibitors are in development including SPHINX, a 3-(trifluoromethyl)anilide scaffold. The objective of this study was to treat two leukaemic cell lines with SPHINX in combination with the established cancer drugs azacitidine and imatinib.

Materials and Methods: We selected two representative cell lines; Kasumi-1, acute myeloid leukaemia, and K562, BCR-ABL positive chronic myeloid leukaemia. Cells were treated with SPHINX concentrations up to 10μM, and in combination with azacitidine (up to 1.5 μg/ml, Kasumi-1 cells) and imatinib (up to 20 μg/ml, K562 cells). Cell viability was determined by counting the proportion of live cells and those undergoing apoptosis through the detection of activated caspase 3/7. SRPK1 was knocked down with siRNA to confirm SPHINX results.

Results: The effects of SPHINX were first confirmed by observing reduced levels of phosphorylated SR proteins. SPHINX significantly reduced cell viability and increased apoptosis in Kasumi-1 cells, but less prominently in K562 cells. Knockdown of SRPK1 by RNA interference similarly reduced cell viability. Combining SPHINX with azacitidine augmented the effect of azacitidine in Kasumi-1 cells. In conclusion, SPHINX reduces cell viability and increases apoptosis in the acute myeloid leukaemia cell line Kasumi-1, but less convincingly in the chronic myeloid leukaemia cell line K562.

Conclusion: We suggest that specific types of leukaemia may present an opportunity for the development of SRPK1-targeted therapies to be used in combination with established chemotherapeutic drugs.

Keywords: acute myeloid leukemia, splice factor kinases, SRPK1, alternative splicing, SPHINX

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INTRODUCTION

Soon after the discovery of pre-mRNA splicing in the late 1970s it became apparent that pre-mRNAs are alternatively spliced so that a multi-exon gene can produce multiple transcripts through exon skipping, intron retention, and the use of alternative 5′ and 3′ splice sites. Over the years it became clear that alternative splicing is a widespread process in eukaryotic cells and that it accounts, to a very

large extent, for the complexity of the proteome (1, 2). The vast majority of human multi-exon genes are alternatively spliced. Population-scale transcriptomic analysis reveals the presence of numerous genetic variants that affect splicing that influence phenotype including disease susceptibility (3). Splice isoforms can exhibit distinct biological properties (pro- or anti-apoptotic; pro- or anti-angiogenic, etc.), and it is now evident that the dysregulation of alternative splicing is implicated in all hallmarks of cancer (4). This presents opportunities for the development of novel cancer therapies. Oncogenic splice isoforms can be targeted directly; alternatively, regulators of alternative splicing can also be targeted.

Alternative splicing is principally regulated by splice factors that bind to specific sequences in pre-mRNA modifying the choice of specific splice sites. One important family of splice factors are the SR proteins. They generally consist of one or more RNA Recognition Motifs (RRMs) and a serine-arginine (SR) rich domain, the latter involved in protein-protein interactions. One of the most widely studied SR protein splice factors is SRSF1, a splice factor with a well-established involvement in cancer (5). The activity of splice factors is in turn, regulated by the activity of splice factor protein kinases. SRSF1 is phosphorylated by the SRPKs (SR protein kinases; SRPK1 and SRPK2 in humans) and CLKs (CDC2-like protein kinases, CLK1-4 in humans (6)). The SRPKs phosphorylate SRSF1 at multiple serines in the SR domain (7). Phosphorylation of SRSF1 by SRPKs in the cytoplasm is required for the accumulation of SR proteins in the nucleus, whereas its phosphorylation by CLKs regulates their association with nuclear speckles and their biochemical activity (8).

The broad developmental and physiological roles of splice factor kinases are not yet fully understood. In the nematode *Caenorhabditis elegans*, the SRPK splice factor kinase SPK-1 is essential for embryogenesis and germline development (8) and inhibits programmed cell death by modifying the alternative splicing of *ced-4*, the *C. elegans* orthologue of human *Apaf-1* (9). The substrates of SRPKs may not be limited to SR proteins, and therefore their functions extend beyond the regulation of alternative splicing. To illustrate their functional complexity, in fertilised mammalian oocytes, SRPK1 catalyses the site-specific phosphorylation of protamines, helping trigger the protamine to histone exchange required for paternal genome reprogramming (10).

Given their involvement in modulating splice factor activity, and in other processes, it is not surprising that splice factor kinase expression is dysregulated in cancer (11), even affecting therapeutic responses to chemotherapy and radiotherapy (12). The acetylation of SRPK1 by the histone acetyltransferase Tip60 alters the activity of SRPK1 and modulates alternative splicing. In cisplatin-resistant breast cancer cells, reduced acetylation of SRPK1 by Tip60 increases its activity, favouring the expression of anti-apoptotic splice variants (13). In breast cancer, elevated SRPK1 activity reduces apoptosis through RBM4-regulated splicing events (14). SRPK1's role in cancer is not limited to the regulation of apoptosis. A migration screen based on a phagokinetic track assay identified SRPK1 as a determinant factor in breast cancer metastasis (15); and a separate study demonstrates that the LIM domain kinase 2 (LIMK2) promotes breast cancer metastasis through SRPK1 activation (16). SRPK1 is implicated in other cancer types, including colorectal cancer and leukaemia (17). SRPK1 is a poor prognostic indicator in colorectal cancer (18) in which it modulates SRSF1-mediated *MKNK2* alternative splicing (19) and is required for the expression of the cancer-associated splice variant RAC1B (20). Of significant recent interest is the work of Tzelepis and colleagues. A CRISPR-Cas9 platform was used to screen for genetic vulnerabilities in acute myeloid leukaemia (AML) and identified SRPK1 as a potential therapeutic target (21). The authors demonstrated that both genetic or pharmacological inhibition of SRPK1 prolonged the survival of murine AML models by altering the splicing of several leukemogenesis-associated genes including *MYB*, *MED24* and *BRD4* (22). Furthermore, SRPK1 has been implicated in other leukaemias, including chronic myeloid leukaemia (CML). Salesse et al. demonstrated that numerous pre-mRNA splicing proteins are overexpressed in patient-derived cell line models of CML, including SRPK1, suggesting that aberrant pre-mRNA splicing may contribute to CML pathogenesis (23). Together, these studies suggest the therapeutic potential of targeting SRPK1 in a variety of cancers, including blood-borne cancers.

To further underline the oncogenic activity of SRPK1, we have previously identified SRPK1 as a key regulator of *VEGFA* alternative splicing (24). SRPK1 promotes the expression of pro-angiogenic VEGFA through the phosphorylation of SRSF1. This causes nuclear accumulation of SRSF1 which then promotes the use of a proximal 3′ splice site in exon 8, favouring the expression of the pro-angiogenic VEGFA splice isoform. Both the knockdown and pharmacological inhibition of SRPK1 shifts the ratio of splice isoforms in favour of the anti-angiogenic isoform of VEGFA165b (24), and we have proposed that targeting SRPK1 could be a viable avenue in prostate cancer (25).

There is considerable interest in developing novel and selective SRPK1 inhibitors. To this end Gammons and colleagues identified a 3-(trifluoromethyl)anilide scaffold named SPHINX that exhibits an IC_{50} of 880 nM for SRPK1 (26). SPHINX has been tested *in vivo* in the context of age-related macular degeneration (AMD), where it convincingly reduces choroidal neovascularization in rodents by promoting the expression of the anti-angiogenic VEGFA splice isoform (27). SPHINX also has potent effects on the growth and spread of orthotopic xenografts of human PC3 prostate cancer cells (25). The aim of the present research was to test the effect of SPHINX on two well-studied leukaemic cell line models (Kasumi-1 and K562) both alone, and in combination with established chemotherapeutic agents, azacitidine and imatinib.

MATERIALS AND METHODS

Cell Culture and SPHINX Treatments

K562 and Kasumi-1 cell lines were purchased from the European Collection of Authenticated Cell Cultures (ECACC) and cultured using RPMI-1640 culture medium with L-glutamine (Sigma

Aldrich, United Kingdom), further supplemented with 10% foetal bovine serum (FBS; Sigma Aldrich) for K562 cells and 20% FBS for Kasumi-1 cells. Cells were used between passages 6–19, seeded at densities of 5×10^5–1×10^6 in T25 flasks and were sub-cultured every 48 h. Cell lines were incubated in 5% CO_2 at 37°C.

K562 and Kasumi-1 cells were treated with SRPK1 specific small molecule inhibitors 5-methyl-N-(2-(morpholin-4-yl)-5-(tri-fluoromethyl)phenyl) furan-2-carboxamide, commonly known as SR Protein Inhibitor X (SPHINX) which was purchased from Enamine (Kiev, Ukraine). SPHINX was dissolved in DMSO (Sigma Aldrich) at a stock concentration of 25 mM. Cells (1×10^6/ml) were seeded in a T25 flask for each treatment which was performed in duplicate. Cells were incubated with 10 nM-10 μM SPHINX for up to 72 h.

Cell Viability and Apoptosis Measurements

Cell counts and viability were determined using either trypan-blue staining and manual counting or using the Luna FL automated cell counter (Logos Biosystems, France). Percentage cell viability was calculated by dividing live cells over the total cell count.

Apoptosis was measured using the CellEvent Caspase-3/7 green detection reagent (ThermoFisher Scientific, United Kingdom) according to manufacturer's instructions. Pelleted cells (20,000) were suspended in reagent and incubated for 45 min at room temperature. Counterstaining was performed with Hoechst for 1 min after which cells were transferred into a cytofunnel (ThermoFisher Scientific) and spun onto a microscope slide using the Cytospin 4 (ThermoFisher Scientific) at 20,000 g for 8 min. Slides were air-dried and mounted using Mowiol aqueous mounting media. Images were taken with a Nikon Eclipse 80i fluorescent microscope at ×40 magnification. For each treatment, green fluorescent cells were considered positive for activated caspase-3/7. For each slide, the total number of caspase positive cells in ten representative fields of view were recorded and calculated as a percentage of the total cells (positive and negative).

SiRNA-Mediated SRPK1 Knockdown

For siRNA-mediated knockdowns, K562 and Kasumi-1 cells were cultured to 80% confluence. Cells were harvested and spun down to remove growth media. For each line, 5×10^5 cells were pelleted and re-suspended in 800 μl of OptiMEM media (Gibco, United Kingdom) and transferred into 6-well plates and incubated in 5% CO_2 at 37°C.

The SRPK1 siRNA (Eurofins, Genomics, United Kingdom) sequence was 5′-UUAAUGACUUCAAUCACUCCAUUGC-3′ and the scrambled siRNA control was 5′GCAGCAGCAGCA GCGGGACTT-3′. Lipofectamine/OptiMEM cocktail (100 μl) (Thermo Fisher Scientific, United Kingdom) was added to 40 μl SRPK1 siRNA (2.5 mM) and incubated at room temperature for 20 min. Following this, siRNA/Lipofectamine/OptiMEM mixture was added to each well at a final concentration of 100 nM and incubated for 4 h, after which 1 ml of culture media was added to each well and cells were incubated for a further 48 h from the time of transfection.

Cell Lysates and Western Blotting

Cell lysates were prepared using RIPA buffer (10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 1% (v/v) Triton X-100, 0.1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS and 140 mM NaCl) supplemented with protease inhibitor tablets (ThermoFisher Scientific). Equal protein samples (20 μg protein), were separated on 10% (v/v) SDS polyacrylamide gels and transferred to PVDF membranes (Sigma Aldrich) which were blocked in 2% (w/v) skimmed milk and probed overnight at 4°C with primary antibodies: anti-SRPK1 (EE-13, Santa Cruz Biotechnology; 1:1000) or anti-pan-SR-1H4 antibody (sc-13509, Santa Cruz Biotechnology; 1:500), anti-β-actin (ab8226, Abcam UK; 1:5000). Membranes were incubated in HRP-linked anti-rabbit or anti-mouse IgG secondary antibody (Cell Signalling; 1:1500) for 1 h at room temperature. Membranes were incubated in Luminata Forte Western HRP substrate (Millipore) for chemiluminescent detection prior to image acquisition which was performed using the LI-COR Odyssey FC imaging system (LI-COR, USA). Images acquired were exported to Image Studio Lite (LI-COR, USA) software for quantification. Experiments were performed in triplicate.

Molecular Characterisation of Kasumi-1 and K562 Cell Lines

Mutation status and protein expression data were downloaded for all AML and CML cell lines available within the Cell Model Passports project, including Kasumi-1 and K562 cells (https://cellmodelpassports.sanger.ac.uk/) (28). Protein expression Z-scores for AML and CML cell lines were plotted as waterfall plots using GraphPad Prism (V9.0.0, GraphPad, United States).

Statistical Analysis

Statistical analyses using ANOVAs or unpaired two-tailed t-tests were performed using GraphPad Prism. Significance levels are indicated by asterisks where $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$. Data are reported as means and error bars show standard error of the means.

RESULTS

Effect of SPHINX on SR Protein Phosphorylation

Two leukaemic cell line models, Kasumi-1 and K562, were selected to investigate the effects of SPHINX. The Kasumi-1 cell line was derived from the peripheral blood of a patient with acute myeloblastic leukaemia (AML); K562 cells were derived from the pleural effusion of a patient with chronic myelogenous leukaemia (CML) and are BCR-ABL positive. The mutation status of SRPKs and SRSF splice factors in Kasumi-1 and K562 cell lines was initially examined using a publicly available dataset (https://cellmodelpassports.sanger.ac.uk/). This revealed no mutations in SRPK1 or SRPK2 in either cell line but demonstrated copy number changes for SRSF2 in Kasumi-1 and SRSF3 in K562. Furthermore, for SRSF12 (in Kasumi-1), these analyses identified a missense mutation (c.536G>A) which causes a replacement of

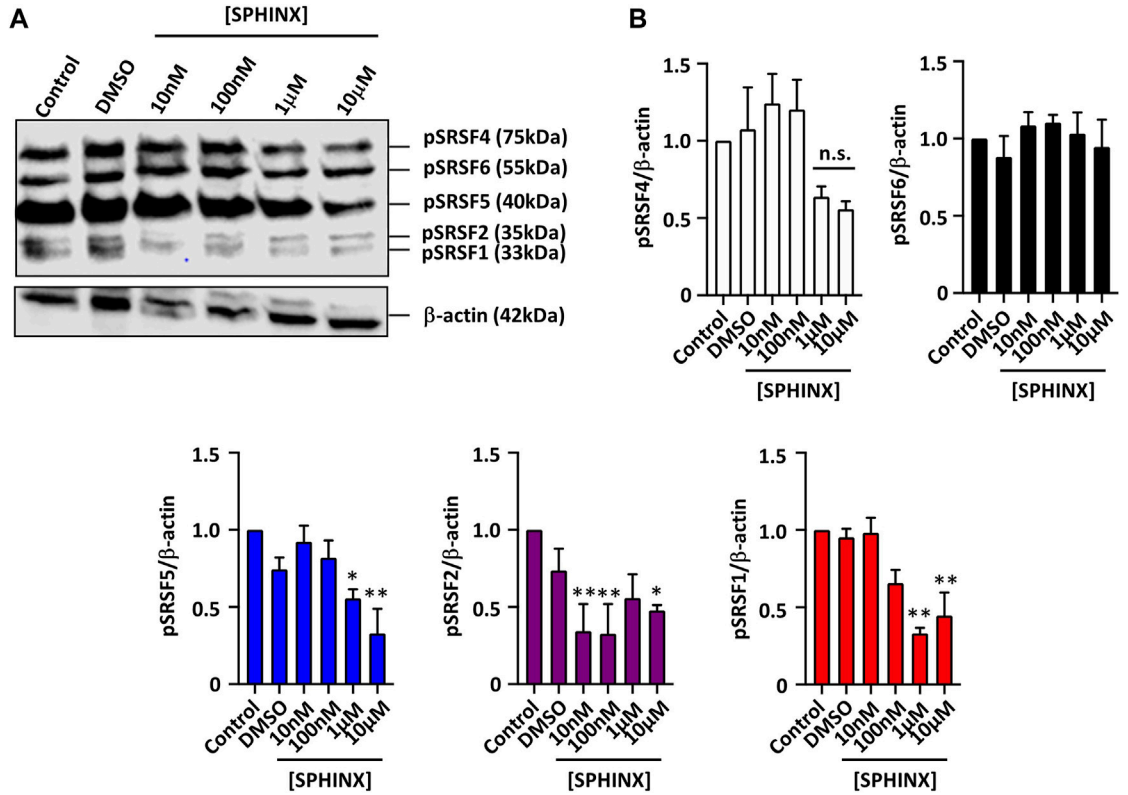

FIGURE 1 | Reduction in phospho-SR protein levels in Kasumi-1 cells treated with SPHINX for 24 h. **(A)** Representative western blot image showing levels of SR protein phosphorylation, and time-matched β-actin. **(B)** Quantification of western blot image normalized with control showing a significant decrease in phosphorylated protein of SRSF5, SRSF2 and SRSF1 at higher concentration of SPHINX (*$p < 0.05$; **$p < 0.01$). One-way ANOVA ($n = 3$).

arginine with glutamine at codon 179 at the protein level (p.R179Q; **Supplementary Figure S1A**). Additionally, the expression of SRPK1 and SRPK2 at the protein level in Kasumi-1 and K562 cells was examined and compared with other AML and CML cell lines available in the Cell Model Passports project (28). This identified that Kasumi-1 cells have comparatively lower levels of SRPK1 and SRPK2 versus K562 cells (**Supplementary Figure S1B**). When comparing SRPK1 and SRPK2 levels across a panel of AML cell lines, Kasumi-1 had the lowest SRPK1 expression and intermediate levels of SRPK2 comparatively (**Supplementary Figure S1C**). Across the CML cell line panel, K562 had comparatively high SRPK1 and SRPK2 levels (**Supplementary Figure S1D**), suggesting that Kasumi-1 and K562 might exhibit differential responses to SRPK1 inhibition by SPHINX.

In order to assess the efficacy of SRPK1 inhibition by SPHINX on substrate phosphorylation, we previously showed that the treatment of PC3 prostate cancer cells with SPHINX led to reduced levels of phosphorylated SR proteins coinciding with increased expression of anti-angiogenic VEGFA (25, 29). SPHINX had the same effect on the leukaemic cell line Kasumi-1 using the mouse monoclonal antibody 1H4 which was specific to phosphorylated SR proteins (**Figure 1A**). Levels of phosphorylated SRSF1, SRSF2, and SRSF5 in Kasumi-1 cells were significantly reduced when cells were exposed to 1–10 μM of SPHINX for 24 h (**Figure 1B**). Conversely, in K562 cells, no change in pSRSF2, pSRSF4 and pSRSF5 protein levels was observed following SPHINX treatment for 24 h (**Supplementary Figure S2**).

Effect of SPHINX on Cell Viability and Apoptosis

Having confirmed the effect of SPHINX on SR protein phosphorylation in Kasumi-1 cells, the effect of a range of SPHINX concentrations on cell viability was next examined. Concentrations ranged from 10 nM to 10 μM, and cell viability was assessed at 24, 48, and 72 h following a single dose of SPHINX (**Figures 2A, C**). In Kasumi-1 cells, 100 nM SPHINX already resulted in significant reductions in cell viability, with further dose-dependent effects on cell viability observed at 1 and 10 μM for 48 and 72 h (**Figure 2A**). However, K562 cells appeared less sensitive to SPHINX, with only a modest reduction in viability at 24 h with 10 μM dosing (**Figure 2C**). To complement these viability analyses, cell death was measured by activated caspase-3/7 staining following SPHINX treatment (**Figures 2B, D**). In Kasumi-1 cells, 100 nM SPHINX increased apoptosis significantly, with dose-dependent effects again observed 72 h following 1 and 10 μM treatments (**Figure 2B**). A significant increase in apoptotic cells was also observed in K562 cells, but only at the higher concentration of 10 μM SPHINX (**Figure 2D**). Together these results suggest that

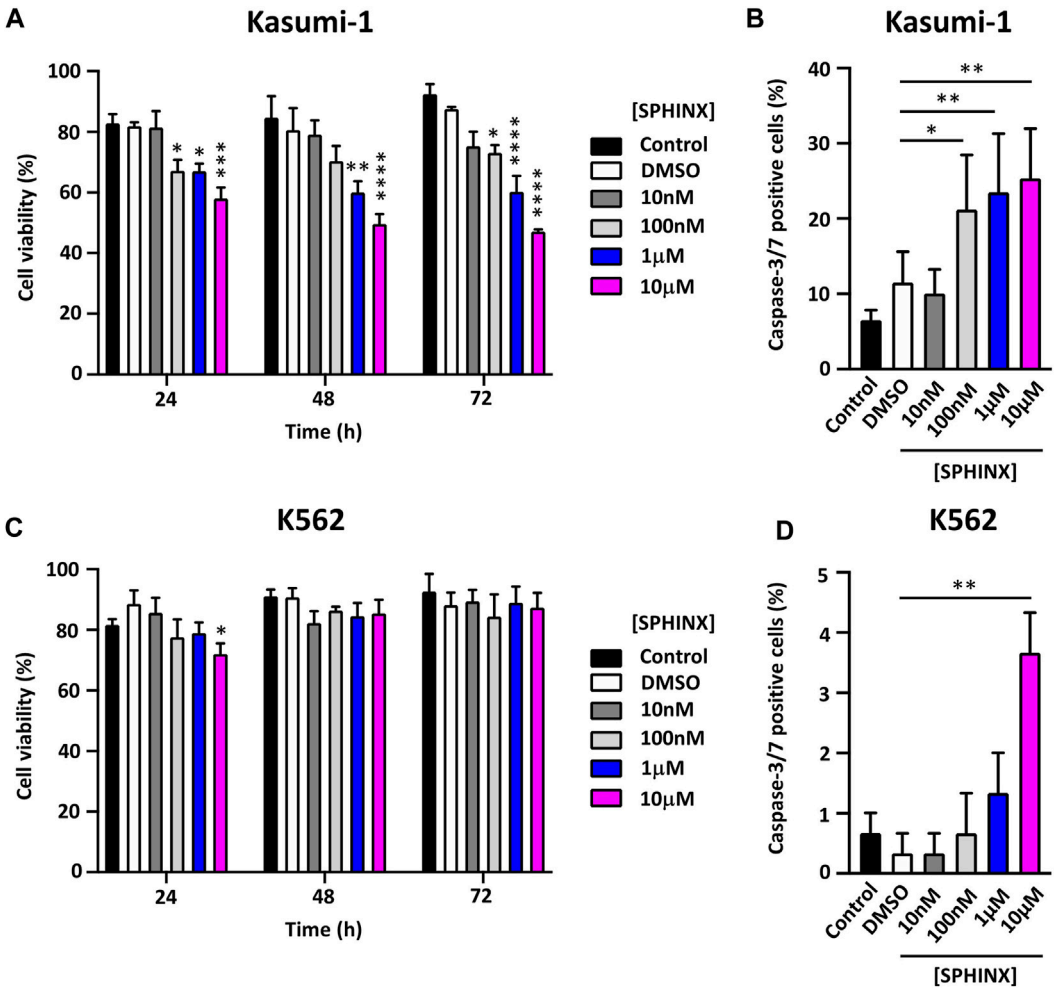

FIGURE 2 | (A) Effect of SPHINX inhibition on cell viability (24 h $^{*}p \leq 0.05$, $^{***}p = 0.0001$; 48 h $^{**}p = 0.0010$, $^{****}p < 0.0001$ and 72 h $^{*}p = 0.029$, $^{****}p < 0.0001$) in Kasumi-1 cells. **(B)** Corresponding increase ($^{*}p = 0.01$; $^{**}p < 0.001$) in caspase-3/7 positive cells at 72 h post-treatment. Cell viability **(C,D)** caspase-3/7 positive cells, K562 cells. Two-way ANOVA ($n = 3$).

Kasumi-1 (AML) cells may be more sensitive to SRPK1 inhibition by SPHINX than K562 (CML) cells.

Effect of siRNA-Mediated SRPK1 Knockdown on Cell Viability

To further examine the sensitivity of the cell lines to the loss of SRPK1 activity, the effect on cell viability of SPHINX treatment was compared with that of siRNA-mediated SRPK1 knockdown. Significant SRPK1 knockdown in both Kasumi-1 and K562 cells was first demonstrated by western blotting (SRPK1-siRNA lanes, **Figures 3A, B, D, E**). In line with our initial findings using SPHINX treatment, SRPK1 knockdown in Kasumi-1 cells significantly reduced cell viability (**Figure 3C**). Interestingly, in contrast to SPHINX treatment, significant effects on K562 cell viability following siRNA-mediated SRPK1 knockdown were also observed (**Figure 3F**). Furthermore, in Kasumi-1 cells, a decrease in pSRSF2, pSRSF4 and pSRSF5 following siRNA-mediated SRPK1 knockdown were also observed (**Supplementary Figure S3A**). Conversely, there appeared to be little change in pSRSF levels in the K562 cells following knockdown (**Supplementary Figure S3B**).

Effect of Combining SPHINX With Azacitidine or Imatinib on Cell Viability and Apoptosis

Previous reports have noted the sensitivity of Kasumi-1 cells to the DNA methyltransferase inhibitor azacitidine (30, 31) and that the tyrosine kinase inhibitor imatinib is effective against BCR-ABL positive K562 cells (32, 33). Kasumi-1 cells were exposed to 325 ng/ml–1.5 μg/ml azacitidine and K562 cells to 3–20 μg/ml imatinib for 24, 48 and 72 h followed by assessment of cell viability. As expected, substantial decreases in cell viability for both cell lines at these drug doses and for all timepoints were observed (**Figures 4A, B**). Next, these treatments were combined

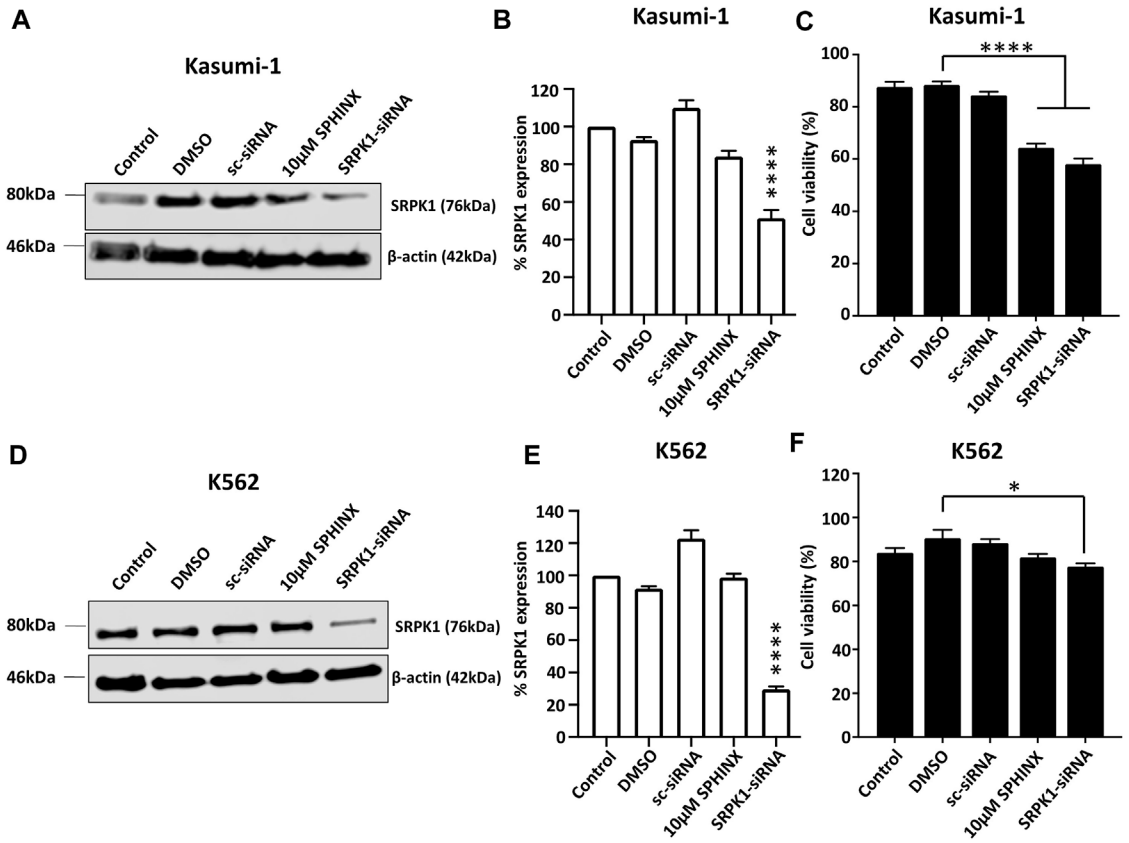

FIGURE 3 | Effects of SPHINX inhibition and siRNA knockdown of SRPK1 in Kasumi-1 and K562 cells. **(A,D)** Representative western blots for Kasumi-1 and K562 cells respectively. Verification of knockdown by western blotting for SRPK1 48 h after siRNA transfection in Kasumi-1 **(B)** and K562 cells **(E)**. Statistical comparisons here are versus control. Corresponding cell viability changes shown in **(C,F)** for Kasumi-1 and K562 cells respectively with statistically significant comparisons indicated. One-way ANOVA (n = 3). **** = $p < 0.0001$.

with 10 μM SPHINX to see if SPHINX potentiated the effects of azacitidine and imatinib (**Figures 4C, D**). As previously shown, Kasumi-1 cells exhibited reduced viability when exposed to 10 μM SPHINX at 24–72 h (**Figure 4C**). When combining 750 ng/ml azacitidine with 10 μM SPHINX in the Kasumi-1 cells, a further decrease in cell viability versus 10 μM SPHINX or 750 ng/ml azacitidine alone was observed (**Figure 4C**). Consistent with earlier findings, the K562 cells did not appear to be sensitive to SPHINX alone and combining 3 μg/ml imatinib with 10 μM SPHINX had no apparent additional effect on cell viability (**Figure 4D**). Finally, the effect of combining SPHINX with azacitidine and imatinib on apoptosis was assessed (**Figures 4E, F**). Consistent with our previous findings, SPHINX alone significantly increased apoptosis in Kasumi-1 cells; this was also observed with azacitidine alone (**Figure 4E**). 10μM SPHINX in combination with 750 ng/ml azacitidine further increased apoptosis in the Kasumi-1 cells. For the K562 cells, 3 μg/ml imatinib monotherapy substantially increased the number of apoptotic cells, with no additive effect on apoptosis when combined with 10 μM SPHINX (**Figure 4F**).

DISCUSSION

In haematological malignancies, mutations have been reported in genes that encode >30 splicing factors with proven or emerging roles in pre-mRNA splicing and its regulation (34). Growing evidence clearly points to the splice factor kinases playing key roles in cancer biology thereby presenting novel and attractive targets for the development of new therapies, potentially combining inhibitors of these splice factor kinases with standard-of-care drug treatments. The splice factor kinases that show promise as targets include the SR protein kinases (SRPKs) and CDC2-like protein kinases (CLKs) (12). Targeting CLKs with the small molecule inhibitor SM09419 in *TP53* mutant AML models results in downregulation of the Wnt signalling pathway and potent anti-tumour effects (35). It therefore seems likely that targeting several splice factor kinases, perhaps with a "cocktail" of inhibitors, could prove to be beneficial in the treatment of AML and other leukaemias, particularly those in which the expression of oncogenic splice isoforms is especially dependent on the activity of splice factor kinases.

Despite the evident promise of targeting splice factor kinases, Wang et al. emphasize an important caveat that applies to many other cancer-associated proteins—namely, that SRPK1 can

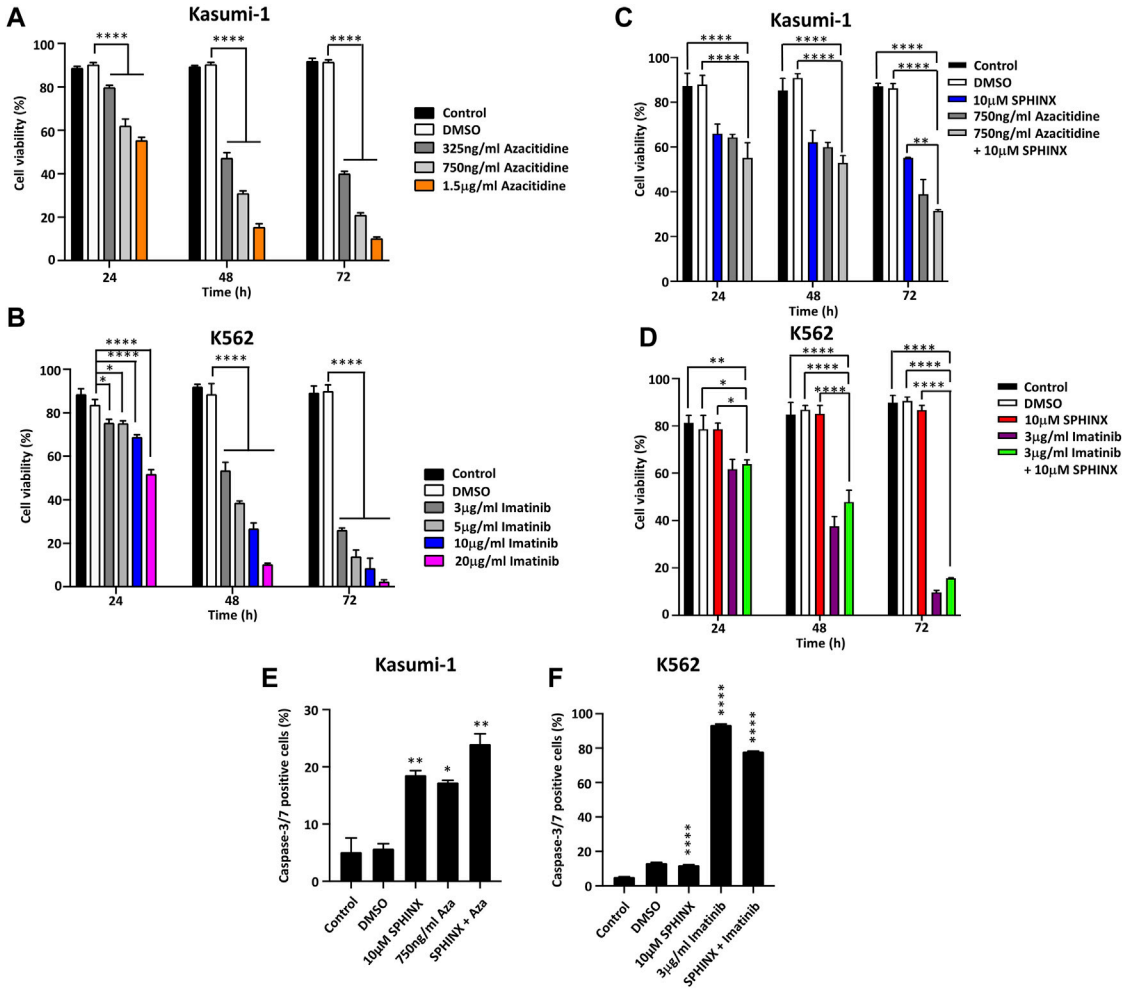

FIGURE 4 | (A) Effects of azacitidine and **(B)** imatinib on cell viability in Kasumi-1 and K562 cells respectively, at 24, 48 and 72 h. The effect on cell viability of combining 10 µM SPHINX with 750 ng/ml azacitidine in Kasumi-1 cells **(C)** and 3 µg/ml imatinib in K562 cells **(D)** at 24, 48 and 72 h. Levels of caspase-3/7 staining 72 h after combined SPHINX and drug treatment in Kasumi-1 cells **(E)** and K562 cells **(F)**. One-way ANOVA ($n = 3$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

potentially act as both an oncogene or tumour suppressor depending on the context. This functional complexity arises through its ability to modulate the activation state of Akt through interaction with the Akt phosphatase PHLPP1 (36). As such, under- or over-expression of SRPK1, can lead to constitutive Akt activation, offering a potential explanation for observations that SRPK1 levels can be downregulated or upregulated in different cancers. Understanding the mechanisms that underpin both activities in human tumours will be important for effective targeting of SRPK1 by cancer therapeutics. To add further complexity, there is another member of the SRPK family in humans, SRPK2, first cloned in 1998 (34). SRPK2 is very similar to SRPK1 in terms of primary sequence, kinase activity and substrate specificity (37). SRPK2 is less well studied, but there is evidence that it is also involved in cancer including leukaemia. In leukaemic cells SRPK2 binds and phosphorylates acinus, an SR protein splice factor, resulting in the upregulation of cyclin A1 expression and increased cell proliferation (38). Therefore, dual therapeutic targeting of SRPK1 and SRPK2 may be necessary to avoid any functional redundancy masking drug effects. To this end, Hatcher and colleagues recently describe SRPKIN-1, a covalent and potent inhibitor of both SRPK1 and SRPK2. SRPKIN-1 efficiently promotes the upregulation of anti-angiogenic VEGFA165b and blocks neovascularisation in a mouse retinal model (39).

In the present study we have focused on evaluating the effect of the SRPK1 inhibitor SPHINX (26) on two well-studied and established cell line models of leukaemia; Kasumi-1, representing acute myeloid leukaemia (AML), and K562 representing chronic myeloid leukaemia (CML). We have observed differential responses in the two cell lines to SRPK1 inhibition by SPHINX. The AML cell line Kasumi-1 appears to be more sensitive to either SRPK1 inhibition by SPHINX or SRPK1 knockdown compared to the CML cell line K562. We also observe that combining SPHINX with established drugs does not augment effects in the case of K562 cells (SPHINX plus imatinib) but appears to enhance the potency of azacitidine in Kasumi-1 cells. Identifying this mechanism of action will form the

basis of future studies. Furthermore, our interesting observation that relative levels of SRPK1 and SRPK2 are anti-correlated with SPHINX sensitivity, i.e., low relative levels of SRPK1/2 equate to SPHINX sensitivity in Kasumi-1, warrants further investigation. Whilst we demonstrate that SPHINX is clearly acting through modulation of SRPK1 and interacting SRSFs, SRPK1 levels themselves may not serve as a robust biomarker of response to SRPK1. Related to our findings in this context, a recent large-scale study examined the sensitivity of hundreds of cancer cell lines to hundreds of drugs, which was correlated with expression of drug targets within the cells. Here, Roy et al. described inverse correlations between target expression and drug sensitivity for 8% of targets, suggesting drug efficacy may not only be determined by expression levels of the drug target, but may also depend on other factors such as genetic background and other molecules that could affect drug-target interactions, including the expression of other gene family members or interacting proteins (40).

There is evidence that mutations that affect the pre-mRNA splicing machinery are especially prominent in AML, and that they are associated with drug resistance through altered splicing of cancer-associated genes, including genes associated with apoptosis (41). We observe a prominent increase in apoptosis in SPHINX-treated AML and CML cells, suggesting that SRPK1 plays a central role in the regulation of alternative splicing, presumably by favouring the expression of anti-apoptotic splice isoforms. This has been observed in breast cancer cells, in which elevated SRPK1 reduces apoptosis through RBM4-regulated alternative splicing (14). SRPK1 activity also appears to counteract apoptosis in colon cancer cells (42) and might therefore be a general mechanism through which SRPK1 is involved in cancer, including AML. The avoidance of apoptosis is a key cancer hallmark, often associated with resistance to chemotherapy. As such, there is significant interest in developing drugs that promote apoptosis in AML, including for example, drugs that target apoptosis regulators such as BCL2 and MCL1 (43, 44).

In summary, we suggest that in the context of AML, and potentially in other types of leukaemia, there may be therapeutic potential in targeting SRPK1 and other splice factor kinases. We envisage that in the future splice factor kinase inhibitors could be used in combination with both well-established and novel drugs for the eventual clinical management of AML treatment.

CONCLUSION

The dysregulation of alternative splicing is a prominent feature of cancer, presenting opportunities for the exploration of novel drug targets. Alternative splicing is regulated by splice factors whose activity is modulated by splice factor kinases. Elevated splice factor kinase activity is observed in several cancer types, including breast, lung and haematological malignancies. The purpose of this study was to examine the effect of the SRPK1 splice factor kinase inhibitor SPHINX on proliferation and apoptosis in two leukaemic cell line models, Kasumi-1 and K562, alone and in combination with the established drugs azacitidine and imatinib.

SPHINX inhibition of SRPK1 reduced the proliferation of and significantly increased rates of apoptosis in the acute myeloid leukaemia cell line Kasumi-1. Kasumi-1 cells are more sensitive to SPHINX than the chronic myeloid leukaemia cell line K562. Combining SPHINX with the clinically-used drug azacitidine potentiated these effects in Kasumi-1 cells. These results highlight the need to continue exploring targeting splice factor kinases such as SRPK1 in leukaemias, particularly in combination with standard-of-care therapies, as well as in other cancers where splice factor kinase activity is elevated.

SUMMARY TABLE

What is Known About This Subject

- Aberrant alternative splicing is implicated in many cancers and plays a prominent role in the development and progression of different types of leukaemia.
- The activity of splice factors is enhanced through their phosphorylation by splice factor kinases that include the SRPKs and CLKs.
- SRPK1 inhibition by SPHINX, a potent and specific inhibitor of SRPK1, modifies the alternative splicing of key cancer associated genes such as *VEGFA*.

What This Paper Adds

- SPHINX reduces cell proliferation and increases apoptosis in leukaemic cell lines.
- The effectiveness of SPHINX is cell-line dependent and therefore some types of leukaemia such as AML may be more sensitive to splice factor kinase inhibition.
- Combining an effective splice factor kinase inhibitor (e.g., SPHINX) with established chemotherapeutic drugs such as azacitidine could potentially augment their clinical effectiveness.

SUMMARY SENTENCE

This work further underlines the importance of targeting the machinery of alternative splicing in leukaemias. There is a need to develop potent and specific splice factor kinase inhibitors.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

CW performed the experiments with assistance from TB. SP and ML were the joint principal investigators and supervisors of CW. RM provided additional supervision and expertise.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/bjbs.2023.11041/full#supplementary-material

Supplementary Figure S1 | (A) Mutation analyses of SRPKs and SRSFs in Kasumi-1 and K562 cells. **(B)** Relative expression (Z-score) of SRPK1 (left) and SRPK2 (right) in Kasumi-1 and K562 cell lines. **(C)** Relative SRPK1 (top) and SRPK2 (bottom) expression (Z-score) in a panel of AML cell lines. Arrows denote Kasumi-1 values. **(D)** Relative SRPK1 (top) and SRPK2 (bottom) expression (Z-score) in a panel of CML cell lines. Arrows denote K562 values. Mutation and protein expression data was downloaded from the Cell Model Passports database (https://cellmodelpassports.sanger.ac.uk/).

Supplementary Figure S2 | Representative western blot image showing levels of SR protein phosphorylation levels in K562 cells treated with SPHINX for 24 h at indicated doses, and time-matched β-actin as loading control.

Supplementary Figure S3 | Effects of SPHINX inhibition and siRNA knockdown of SRPK1 in Kasumi-1 and K562 cells on pSRSF protein levels. **(A,B)** Representative western blots for Kasumi-1 and K562 cells respectively, with β-actin as loading control.

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ORIGINAL RESEARCH
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Isocitrate Dehydrogenase *IDH1* and *IDH2* Mutations in Human Cancer: Prognostic Implications for Gliomas

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Background: There are isolated reports of mutations in genes for isocitrate dehydrogenases (*IDH1* and *IDH2*), but few have been examined in a large number of different malignancies. We aimed to analyze mutational prevalence of these genes in a large series of cancers and determine their significance in most mutated phenotype.

Methods: We analyzed the frequencies of *IDH1* and *IDH2* mutations in 14,726 malignancies of 37 cancers. Furthermore, we examined these mutations in the most frequent cancer (gliomas, 923 cases) from a single cohort, and determined their clinical significance.

Results: *IDH1* mutations were present in 3% (473/14,726) of cancers. The highest frequencies were in oligodendrogliomas (91/102, 89%), anaplastic oligodendrogliomas (40/46, 87%), and diffuse astrocytomas (89/116, 77%). *IDH2* mutation was detected in <1% (83/14,726) of cancers, but were present in 13% (6/46) of anaplastic oligodendrogliomas, 9% (9/102) of oligodendrogliomas, and in 5% (2/39) of cutaneous squamous cell carcinomas. Further analyses of 923 gliomas revealed 34 and 1% of *IDH1* and *IDH2* mutations, respectively. In up to 342 months of follow-up, *IDH1* and *IDH2* mutations were significantly linked with better overall (OS) (both $p = 0.01$) and progression-free survival (PFS) ($p = 0.01$; $p = 0.004$), respectively.

Conclusion: *IDH1* and *IDH2* are often mutated in a tissue-specific manner, most commonly in gliomas. Mutation in both genes is linked to OS and PFS. Our findings suggest that these genes are promising therapeutic targets and strong prognostic biomarkers in gliomas.

Keywords: *IDH1*, *IDH2*, mutation, cancer, glioma, dehydrogenase, isocitrate

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INTRODUCTION

Malignancy is the most common non-communicable disease and accountable for one in eight deaths across the globe. The incidence of cancer and cancer-related death for the year 2021 has been projected to occur in 1,898,160 and 608,570 cases, respectively in the United States. The cancer-related death rate has significantly fallen since 2017, accounting for an overall decline of 29% that is equal to 2.9 million fewer deaths. Over the past decade, the death rate was shown to be significantly decreased in the leading cancers including lung, colorectal, breast, and prostate nevertheless, the decline slowed in breast and colorectal cancers of females, and the decline stopped in prostate cancer

TABLE 1 | Human cancer samples (solid tumors) analyzed for *IDH1* and *IDH2* gene mutations.

S. No	Name of organ	Type of cancer	Number of samples
1	Adrenal gland	Adrenocortical carcinoma	92
2	Ampulla of vater	Ampullary carcinoma	160
3	Biliary tract	Colangiocarcinoma	195
4	Bladder	Bladder cancer	413
5	Bowel	Colorectal adenocarcinoma	619
6	Breast	Breast cancer	2,509
7	Brain	Glioma	1,004
8		Glioblastoma	543
9		Medulloblastoma	125
10	Cervix	Cervical squamous cell carcinoma	297
11	Esophagus	Esophageal squamous cell carcinoma	139
12		Esophageal carcinoma	559
13		Gastric adenocarcinoma	78
14		Metastastic esophagogastric cancer	341
15		Esophageal adenocarcinoma	182
16	Stomach	Stomach adenocarcinoma	440
17	Eye	Uveal melanoma	80
18	Head and Neck	Head and neck squamous cell carcinoma	523
19		Oral squamous cell carcinoma	40
20		Nasopharygeal carcinoma	56
21	Kidney	Kidney renal clear cell carcinoma	446
22	Liver	Hepatocellular Adenoma	46
23		Hepatocellular carcinomas	243
24	Lung	Small cell lung cancer	120
25		Non-small cell lung cancer	447
26	Ovary	Ovarian serous cystadenocarcinoma	489
27	Pancreas	Pancreatic adenocarcinoma	456
28	Peripheral nervous system	Pediatric neuroblastoma	1,089
29	Pleura	Pleural mesothelioma	22
30	Prostate	Metastatic prostate adenocarcinoma	444
31		Prostate adenocarcinoma	1,465
32	Skin	Basel cell carcinoma	293
33		Cutaneous squamous cell carcinoma	39
34		Metastatic melanoma	110
35		Skin cutaneous melanoma	448
36	Testis	Germ cell tumors	180
37	Thymus	Thymoma	123
38	Uterus	Uterine corpus endometrial carcinoma	373
39		Uterine carcinosarcoma	57
40	Vulva	Squamous cell carcinoma of the vulva	15
		Total	15,300[a]

[a]*Derived from 14,726 patients.*

(1). Various next-generation transcriptomic, genomic, and proteomic studies identified many genetically deregulated genes in human cancer. These altered cancer gene clusters exert deregulated signaling on certain pathways as somatic mutations in receptor tyrosine kinases (RTKs) and their downstream pathway members including different RAS (H/K/N) molecules constitutively trigger canonical mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) signaling. Particularly, high frequent genetic alterations of various genes like *TP53*, *BRAF*, *RAS*, *EGFR*, *PIK3CA*, *PTEN*, *HER2*, *UDX*, *ALK*, *TERT*, *mTOR*; *IDH1*, etc. were documented in the oncogenesis of several kinds of human malignancies (2–17). Aberrant activation of the vital pathways promotes uncontrolled cell division, proliferation, growth, invasion, and metastasis that collectively leads to tumorigenesis.

Isocitrate dehydrogenase 1 (*IDH1*) gene mutations were high frequently detected in human cancers particularly in secondary glioblastomas (>70%) (18). IDH1 mutations are present mainly in the hotspot arginine at codon R132 and many different *IDH1* mutations (R132H, R132S, R132C, and R132G) were also reported for the residue R132. The *IDH2* mutations were identified in codon 172 and malignancies with no mutations in *IDH1* frequently showed mutations in the cognate amino acid arginine (R) at 172 of the *IDH2* gene. All the codon R132 *IDH1* mutants were shown to decrease the enzymatic activity of the IDH1 (16). Frequent mutations of the *IDH1* have also been identified in hormone receptor-positive (HR+) breast adenocarcinoma, thyroid cancer, cholangiocarcinoma (50–70%), and *IDH2* (R172S) in benign giant cell tumors of the bone (80%) (17, 19–22).

IDH plays a key role within the Krebs cycle and produces alpha-ketoglutarate (α-KG) by catalyzing the oxidative decarboxylation of isocitrate. The IDH activity is exclusively dependent on nicotinamide adenine dinucleotide phosphate

TABLE 2 | Mutational prevalence of *IDH1* and *IDH2* genes in various human cancers.

Type of cancer	Mutational prevalence of *IDH1*[a]	Type of cancer	Mutational prevalence of *IDH2*[a]
Oligodendroglioma	89.2% (91/102)	Anaplastic Oligodendroglioma	13.0% (6/46)
Anaplastic Oligodendroglioma	87.0% (40/46)	Oligodendroglioma	8.8% (9/102)
Diffuse Astrocytoma	76.7% (89/116)	Cutaneous Squamous Cell Carcinoma	5.1% (2/39)
Anaplastic Astrocytoma	54.1% (86/159)	Skin Cancer, Non-Melanoma	3.4% (10/293)
Intrahepatic Cholangiocarcinoma	29.1% (46/158)	Lung Squamous Cell Carcinoma	2.6% (4/155)
Glioblastoma Multiforme	5.6% (45/796)	Intrahepatic Cholangiocarcinoma	2.5% (4/158)
Extrahepatic Cholangiocarcinoma	5.4% (2/37)	Uterine Endometrioid Carcinoma	2.0% (4/200)
Cutaneous Squamous Cell Carcinoma	5.1% (2/39)	Colorectal Adenocarcinoma	1.8% (11/619)
Cutaneous Melanoma	4.9% (27/550)	Uterine Carcinosarcoma	1.7% (1/57)
Skin Cancer, Non-Melanoma	3.7% (11/293)	Diffuse Astrocytoma	1.7% (2/116)
Esophagogastric Adenocarcinoma	2.5% (2/80)	Tubular Stomach Adenocarcinoma	1.3% (1/79)
Uterine Serous Carcinoma	2.3% (1/44)	Anaplastic Astrocytoma	1.3% (2/159)
Bladder Urothelial Carcinoma	2.2% (9/412)	Esophagogastric Adenocarcinoma	1.2% (1/80)
Colorectal Adenocarcinoma	2.1% (13/619)	Hepatocellular Carcinoma	1.2% (3/243)
Adenocarcinoma of the Gastroesophageal Junction	1.7% (1/57)	Esophagogastric Cancer	1.1% (4/347)
Uterine Endometrioid Carcinoma	1.5% (3/200)	Esophageal Squamous Cell Carcinoma	0.9% (3/324)
Stomach Adenocarcinoma	1.3% (4/308)	Esophageal Adenocarcinoma	0.76% (2/303)
Tubular Stomach Adenocarcinoma	1.3% (1/79)	Glioblastoma Multiforme	0.5% (4/796)
Esophagogastric Cancer	1.1% (4/347)	Bladder Urothelial Carcinoma	0.5% (2/412)
Thymoma	0.8% (1/123)	Cervical Squamous Cell Carcinoma	0.4% (1/247)
Head and Neck Squamous Cell Carcinoma	0.8% (4/515)	Prostate Adenocarcinoma	0.4% (7/1909)
Ampullary Carcinoma	0.6% (1/160)	Cutaneous Melanoma	0.4% (2/550)
Esophageal Squamous Cell Carcinoma	0.6% (2/324)	Stomach Adenocarcinoma	0.3% (1/308)
Renal Clear Cell Carcinoma	0.5% (2/426)	Head and Neck Squamous Cell Carcinoma	0.2% (1/515)
Prostate Adenocarcinoma	0.4% (8/1909)		

[a]*% (Mutated samples/Analyzed total samples) of the indicated cancer type.*

(NADP+) which is catalyzed by IDH1 to produce NADPH that is involved in controlling oxidative damage of a cell (23). The cancer-associated *IDH1* mutations have been demonstrated to produce 2-hydroxyglutarate as this IDH (IDH1 and IDH2) mutant enzyme carries a neomorphic catalytic function and converts alpha-ketoglutarate to 2-hydroxyglutarate that suppresses the histone lysine demethylases (23–25). It has been reported that an *IDH1* mutation was potentially able to form glioma hypermethylation phenotype while *IDH2* could promote acute myeloid leukemia (26). Nevertheless, a prominent status of the *IDH1* and *IDH2* mutations has never been undertaken particularly within a large number of different human cancer samples.

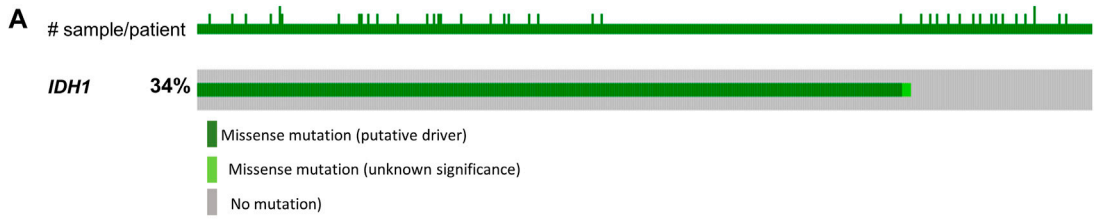
Given the importance of these genes, we investigated the *IDH1* and *IDH2* mutations in a large series of cancer cases (n = 14,726) (solid malignancies) from the data of The Cancer Genome Atlas and the Memorial Slone Kettering Cancer Centre.

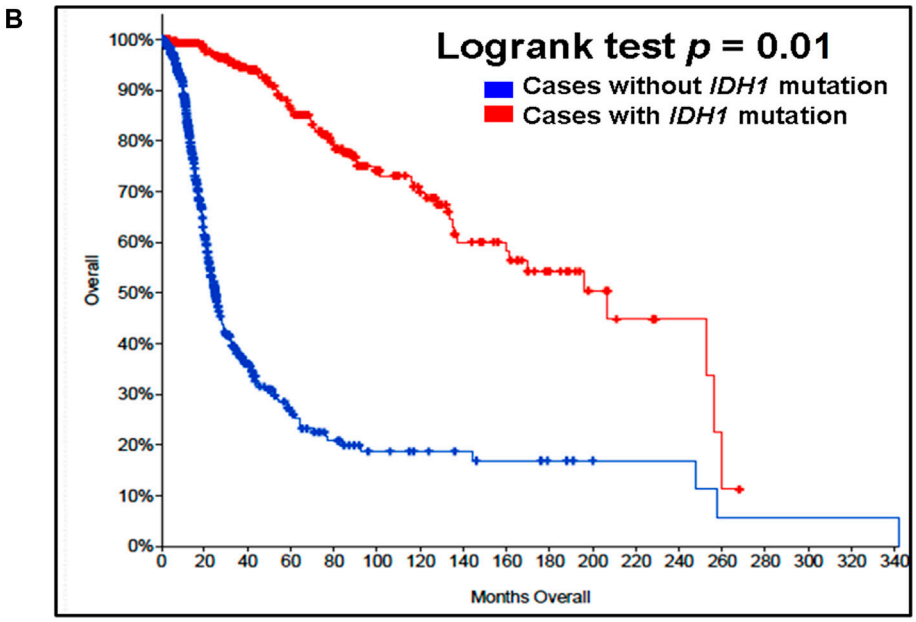
METHODS

As detailed in **Table 1**, we analyzed 15,300 malignant tumour samples (obtained from 14,726 patients) of 37 different types of malignancies in solid tissue cancers. All data was derived from The Cancer Genome Atlas studies by performing various analyses using the methods within the cBioPortal (www.cbioportal.org), an open-access, open-source, and publicly available platform for interactive exploration of multidimensional cancer genomics datasets. Approval statement/informed consent is not required for this study as we used data from a publicly available database.

We explored the cBioPortal database for mutational analysis of *IDH1* and *IDH2*. In brief, firstly we selected one/two cohorts of larger sample size against each malignancy from the different cohorts (cancer studies) available in the datasets (accessible from the homepage of cBioPortal). In total, 40 different cohorts were selected (users have the option to select one or more than one cohort). Secondly, we selected only "mutation" under the genomic profile menu (on the same page). Other genomic profiles including the copy number variants (CNVs), structural variant, RNAseq, etc., were excluded (unselected). Thirdly, we selected "samples with mutation data" under the patient/case set menu. Fourthly, we input *IDH1* or *IDH2* under the "enter genes menu" (users have options to enter multiple genes) and queried the *IDH1*/*IDH2* against the selected set of malignancies. The query generated a newer window with multiple tool tab options such as oncoprint, cancer types summary, plots, mutations, survival, etc., to visualize the results against various parameters of selected cohorts. Users may perform different analyses of selected datasets by selecting and submitting the intended (any of the above-indicated) tool tab. We utilized the tool tab "cancer types summary" to visualize and obtain the prevalence of *IDH1*/*IDH2* mutations in various selected malignancies. Complete and comprehensive step-by-step procedures are clearly described previously for mining the cBioPortal database (27).

The Memorial Sloan Kettering Cancer Centre data of 1,004 glioma samples (derived from 923 patients) was analyzed for *IDH1* and *IDH2* mutations within the cBioPortal (www.cbioportal.org), and clinical links including overall survival (OS) and progression-free survival (PFS) was performed by





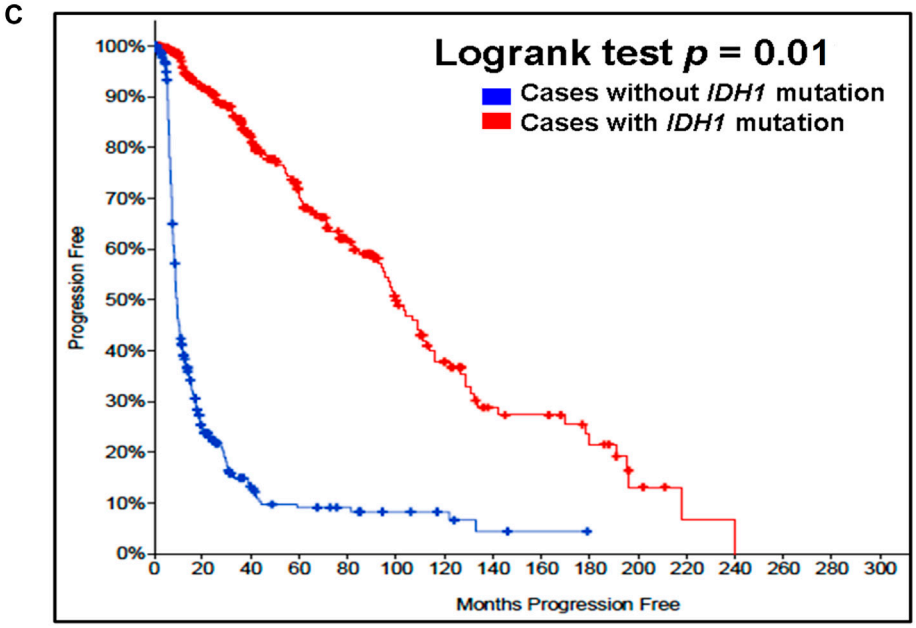


FIGURE 1 | Prevalence and prognostic significance of *IDH1* mutations in gliomas **(A)** *OncoPrint tab*. The tab shows the *IDH1* mutations identified in gliomas. The row indicates the *IDH1* gene and each column show a tumor sample. The green squares plotted on the columns show non-synonymous somatic mutations. **(B)** *Overall survival curve*. Of 923 glioma cases, 2 patients were excluded from survival analysis due to overlap. The total number of patients included in the overall survival analysis = 921. Number of cases with *IDH1* mutation = 312 (number of events = 64; median overall survival (months) = 207). Number of cases without *IDH1* mutation = 609 (number of events = 280; median overall survival (months) = 25), $p = 0.01$. **(C)** *Progression-free survival curve*. The total number of patients included in the overall

(*Continued*)

FIGURE 1 | progression-free survival analysis = 622. Number of cases with *IDH1* mutation = 302 (number of events = 116; median progression-free survival (months) = 100). Number of cases without *IDH1* mutation = 320 (number of events = 262; median progression-free survival (months) = 9), $p = 0.01$. Diagrams **(B,C)** display the Kaplan–Meier plot of overall survival and progression-free survival of glioma patients in the absence or presence of the *IDH1* mutations which is indicated in blue and red colour, respectively.

selecting the tool tab "survival tab," excluded the overlapping samples and results were visualized as described earlier (27).

Statistical genomic analyses were executed utilizing the methods incorporated within the cBioPortal (www.cbioportal.org) (27). Kaplan–Meier plots with a log-rank test were performed to determine the OS and PFS of gliomas presence of a minimum of one mutation or absence of mutation in the related queried candidate gene. $p < 0.05$ was regarded as statistically significant.

RESULTS

The composition of the 15,300 different cancer samples and their malignant types are shown in **Table 1**. As detailed in **Table 2**, overall, 3% (473/14,726) of solid tumour cancer cases harbored mutations in *IDH1*. The highest frequencies were present in oligodendrogliomas, anaplastic oligodendrogliomas, and diffuse astrocytomas. The overall prevalence of *IDH2* mutation was <1% (83/14,726). The highest frequencies were present in anaplastic oligodendrogliomas, oligodendrogliomas, and cutaneous squamous cell carcinomas. These results clearly indicate that *IDH1* and *IDH2* are often mutated in solid cancers in a tissue-specific manner and the mutational incidence was frequent mainly in gliomas, suggesting that *IDH1* and *IDH2* may play important roles in these types of solid human cancer.

Our analysis of cancer data from The Cancer Genome Atlas revealed a high incidence of *IDH1* and *IDH2* mutations in subtypes of gliomas. Thus, to determine the importance of these gene mutations in glioma, we analyzed them in a single and a large cohort of glioma from the Memorial Sloan Kettering Cancer Centre data sets and also examined the association of mutations of these genes in the prognosis of gliomas. As seen in **Figure 1A**, *IDH1* harbored somatic mutations overall in 34% (314/923) of gliomas, whilst its presence significantly predicts an improved overall survival (OS) and better progression-free survival (PFS) (**Figures 1B,C**). The *IDH2* mutation was detected overall in 2.5% (23/923) of gliomas (**Figure 2A**). The *IDH2* mutation-bearing patients were also statistically significantly associated with improved OS and better PFS (**Figures 2B,C**).

DISCUSSION

IDH1 and *IDH2* are recurrently mutated in several types of human cancer (15–17, 19–22). Particularly, point mutations of these genes are major therapeutic targets and important prognostic markers in brain tumors (15, 16). Nonetheless, the prevalence of these mutations has not been analyzed in a large number of different types of human cancer despite the availability of next-generation sequencing data. Therefore, we mined the human cancer data derived from 37 different types of cancers, finding a high rate of *IDH1* and *IDH2* mutations in a tissue-specific manner mainly in gliomas suggesting a role for these genes in carcinogenesis. Our analysis revealed that the overall prevalence of *IDH1* mutations in cancer was 3%. The oligodendrogliomas showed a high rate of *IDH1* mutation, followed by anaplastic oligodendrogliomas and diffuse astrocytomas (all > 75%). In contrast, the overall prevalence of *IDH2* mutation was 1%, being most frequent in anaplastic oligodendrogliomas, oligodendrogliomas and cutaneous squamous cell carcinomas (<15%). Even though a number of studies were combined and cases were different in each study in our analysis, *IDH1* or *IDH2* mutational frequencies of our study reflect previously published results (16).

Having found a high frequency of *IDH1* and *IDH2* mutations in subtypes of gliomas, we hypothesised role in long-term follow-up studies, finding that both *IDH1* and *IDH2* mutations are strong prognostic factors, supporting other data (15, 16). Importantly, it has been shown that *IDH* mutations are an independent prognostic marker of favorable outcomes (28). Thus, *IDH* mutations have been shown to be associated with longer survival, unlike a mutated *TP53* cases (29). The *IDH1* mutation has been shown to be a stand-alone favorable prognostic element in low-grade oligodendrogliomas (LOs), anaplastic oligodendrogliomas (AOs) particularly when the *TP53* is not overexpressed (30). Moreover, a study investigated the prognostic value of *IDH* mutations in 99 secondary high-grade gliomas revealed that an *IDH* mutation did not associate with increased PFS although secondary anaplastic glioma patients with *IDH* mutation showed a significantly improved outcome (31). A previous study performed a meta-analysis of *IDH1* and *IDH2* mutations from 55 different studies with 9,487 glioma tumors found both mutations were independently and statistically significantly associated with better OS and PFS of glioma patients (32). Analyses of 24 different studies displayed that glioma patients with *IDH* mutations were associated with improved OS and PFS (33). These results provide additional evidence that collectively indicates the important roles of these genes and suggests that *IDH* mutations are strong prognostic markers for survival in gliomas. Conversely, recently it has been shown that the median overall survival from the first progression was not significantly different between the IDH1 mutant and wild-type group when primary and secondary glioblastomas were combined. On the other hand, the median overall survival from the initial diagnosis was significantly different (34). These findings clearly indicate that *IDH1* and *IDH2* could serve as potential independent diagnostic and prognostic biomarkers in these malignancies.

IDH1 and *IDH2* are promising molecular targets for precision therapy not only in gliomas but also in other malignancies.

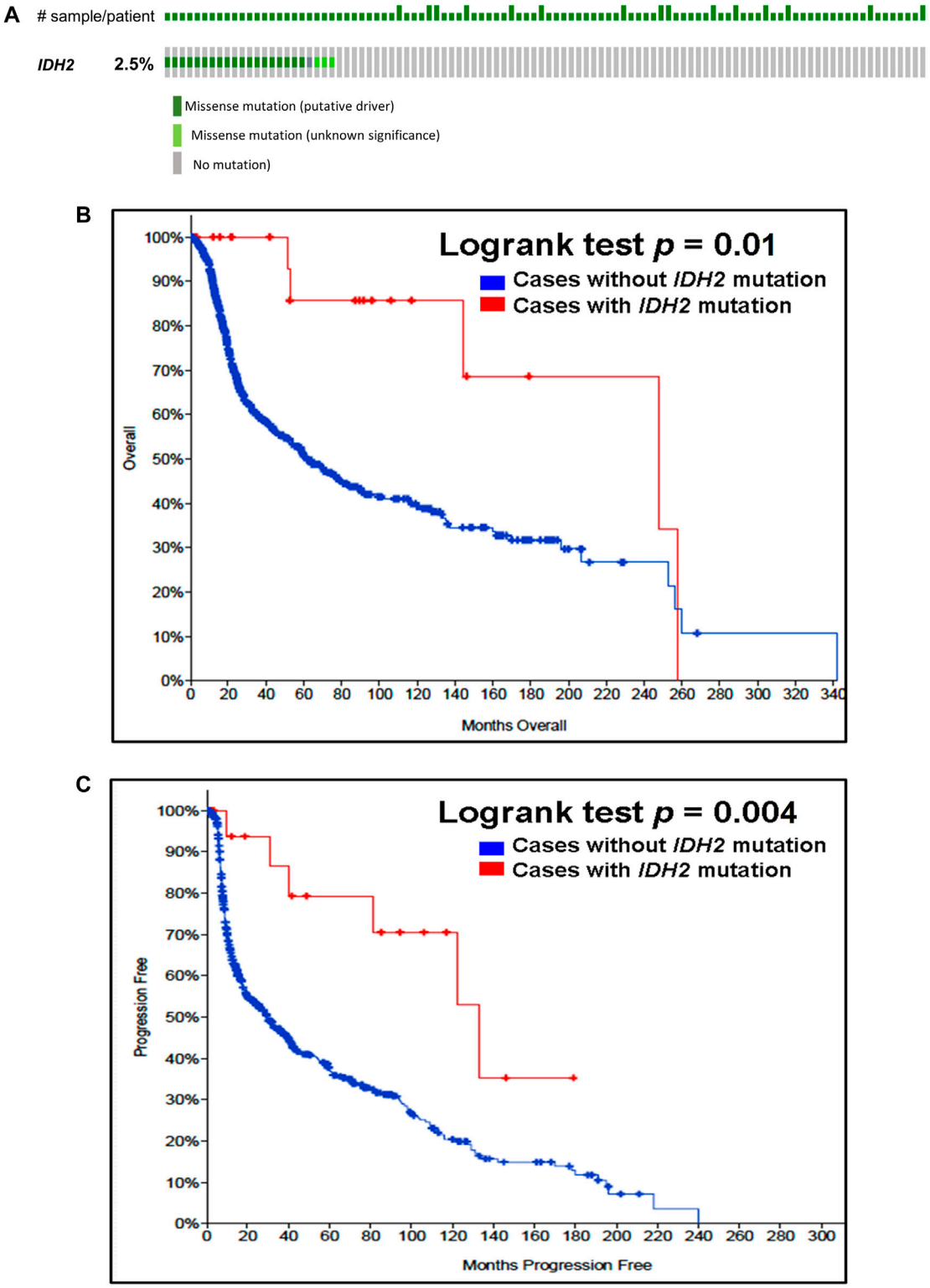


FIGURE 2 | Prevalence and prognostic significance of *IDH2* mutations in gliomas **(A)** *OncoPrint tab*. The tab shows the *IDH2* mutations found in gliomas. The row indicates the *IDH2* gene and each column shows a tumor sample. The green squares plotted on the columns show non-synonymous somatic mutations. **(B)** *Overall survival curve*. The total number of patients included in the overall survival analysis = 923. Number of cases with *IDH2* mutation = 23 (number of events = 5; median overall survival (months) = 248). Number of cases without *IDH2* mutation = 900 (number of events = 339; median overall survival (months) = 62), $p = 0.01$. **(C)** *Progression-free survival curve*. The total number of patients included in the overall progression-free survival analysis = 623. Number of cases with *IDH2* mutation = 20 (number of

(*Continued*)

FIGURE 2 | events = 6; median progression-free survival (months) = 133). Number of cases without *IDH1* mutation = 603 (number of events = 372; median progression-free survival (months) = 30), $p = 0.004$. Diagrams **(B,C)** display the Kaplan–Meier plot of overall survival and progression-free survival of glioma patients in the absence or presence of the *IDH2* mutations which are indicated in blue and red colour, respectively.

Consistent with this notion, the mutant IDH1 (ivosidenib) and IDH2 (enasidenib) protein inhibitors have been initially approved by the U.S. food and drug administration (FDA) for relapsed/refractory acute myeloid leukemia (AML)-bearing *IDH1* and *IDH2* mutations (35). Subsequently, IDH1 inhibitor was also approved for newly diagnosed cases of AML, and currently the drug is being clinically evaluated for other cancers including cholangiocarcinoma with *IDH1* mutation (35, 36). Furthermore, the *IDH1*-mutated tumors were recently targeted by a vaccine that exhibited vaccine-mediated tumor response in the majority of cases (37). Particularly, the FDA has already approved the mutant *IDH1* and *IDH2* test and hence, these findings can be expanded by testing *IDH1* and *IDH2* mutations in different malignancies in both the diagnostic phase and during the course of treatment to examine if the mutation evolves so that the tumors harboring *IDH1* and *IDH2* mutation could benefit from the *IDH1/2*-mediated targeted therapy. These advances collectively demonstrate that the *IDH1* and *IDH2* mutations play a key role in the therapeutic determination of gliomas and a subset of other malignancies.

In conclusion, we identified a high incidence of *IDH1* and *IDH2* mutations in a tissue-specific manner most notably in gliomas, and various types of skin cancer suggesting a potential role in the pathogenesis of these solid malignancies. Thus, *IDH1* and *IDH2* could be useful as molecular therapy targets. Furthermore, patients bearing the *IDH1* mutation can be benefitted from the ivosidenib or recently developed IDH1 mutant-specific peptide vaccine (IDH1-vac) and may also serve as diagnostic markers in these cancers. The *IDH1* and *IDH2* gene mutations can be used in clinical practice as strong prognostic biomarkers in gliomas as they could predict better survival.

This work represents an advance in biomedical science because it shows *IDH1* and *IDH2* mutational spectrum, significant prevalence in large cancer series and benefit of testing them for prognosis and therapeutic management.

SUMMARY TABLE

What Is Known About This Subject?

- Isocitrate dehydrogenase genes, *IDH1* and *IDH2* have been demonstrated to be altered in brain cancers
- The *IDH1* and *IDH2* mutations were shown to predict the outcome of the patients with various brain malignancies
- Mutant IDH1 bearing gliomas can be therapeutically benefited from recently developed IDH1 mutant-specific peptide vaccine

What This Work Adds

- Analyses of *IDH1* and *IDH2* mutations in large malignant series shows a complete spectrum of these mutations in human cancers
- The *IDH1* and *IDH2* somatic mutations play a significant role not only in brain tumors but also in other malignancies

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AM conceived and conducted the study, collected and analyzed the data and wrote the manuscript. AA critically reviewed the data and the manuscript. AM and AA finalized and approved the manuscript.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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