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REVIEW

Immunotherapy – new perspective in lung cancer

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Abstract

Lung carcinoma is associated with a high mortality worldwide, being the leading cause of cancer death. It is mainly classified into squamous non-small cell lung cancer (NSCLC), non-squamous NSCLC, and small cell lung cancer. However, such malignancy has been increasingly subdivided into histological and molecular subtypes to guide treatment. Therapies can be used in adjuvant and palliative settings. Regarding immunotherapy, it has been widely tested in both first or subsequent palliative lines. In this sense, drugs such as pembrolizumab, nivolumab, atezolizumab, ipilimumab, avelumab, and durvalumab have been assessed in large studies. Some of these trials have also studied these medicines in adjuvant and in maintenance therapy. In recent years, advances in immunotherapy have raised the hope that the unfavorable prognosis observed in several affected individuals can be changed. Immunotherapy has increased the overall survival in squamous NSCLC, non-squamous NSCLC, and small cell lung cancer. However, it has added to the oncology practice some side effects that are unusual in standard chemotherapy and require special clinical support. In order to show how immunotherapy is being applied in the treatment of lung carcinoma, we reviewed the main studies in adjuvant and palliative scenarios. What is the better scheme? What is the better combination? What is the better dose? When should we use immunotherapy? Does programmed cell death ligand 1 expression significantly interfere in immunotherapy efficiency? Some of these questions have already been answered, while others require more investigations.

Key words: Lung cancer; Treatment; Immunotherapy; Squamous non-small cell lung cancer; Non-squamous non-small cell lung cancer; Small cell lung cancer

Core tip: Immunotherapy has represented a new hope in the treatment of lung cancer. Improvements in global survival curves in metastatic disease and in local advanced disease have been observed with that therapeutic modality. However, some side effects

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that are unusual in standard chemotherapy have been frequently observed in immunotherapy, and they require special clinical support. This review aims to discuss some aspects regarding immunotherapy in non-small cell lung cancer and the perspectives about the use of this treatment in the adjuvant scenario and in small cell lung cancer.

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INTRODUCTION

The immunotherapy was thought in the 19th century by Coley in the treatment of sarcoma in patients with erysipelas[1]. In the 20th century, the idea of immunologic approaches in cancer treatment was presented and has been evaluated in different tumors. Interestingly, in 1986, interferon-alpha was approved for hairy-cell leukemia[2], and, in the 90's, BCG was approved for adjuvant setting in bladder cancer. Furthermore, interleukin-2 was a new approach for metastatic melanoma in 1993[3]. However, during 20 years the immunotherapy was forgotten, arising in 2011 with the approval of anti-cytotoxic T-lymphocyte antigen 4 for metastatic melanoma[4].

Lung cancer is the first cause of death in oncology and the past global survival curve used to be poor. The revolution provided by immunotherapy in oncology has represented a new hope in the treatment of this disease. But new different adverse effects have also been observed accompanying these advances, most of them immune-mediated[5]. In addition, improvements in global survival curves in metastatic disease and local advanced disease have been noted with immunotherapy[6]. In this review, we discuss some aspects regarding immunotherapy in non-small cell lung cancer (NSCLC) and some perspectives about the use of this therapeutic modality in the adjuvant scenario and in small cell lung cancer (SCLC).

IMMUNOTHERAPY AS FIRST LINE IN METASTIC NON-SQUAMOUS NSCLC

Immunotherapy with checkpoint inhibitors has become the standard treatment for advanced nondriver-mutated non-squamous NSCLC, mainly with anti-programmed cell death protein 1 and anti-programmed cell death ligand 1 (anti-PD-L1) and, in a few cases, with anti-cytotoxic T-lymphocyte antigen 4. The selection of the adequate therapy for each patient is guided by a proper immunohistochemistry, according IALSC Atlas of PD-L1 immunohistochemistry testing in lung cancer (2017)[5]. It is important to be highlighted that the above-mentioned analysis allows the stratification of patients in 3 groups of PD-L1 expression: < 1%, 1%-49%, and > 50%, and this classification correlates with overall rate response to treatment[6]. Nowadays, various challenges have been faced in the anatomopathological examination scenario. Increasingly, biopsies have been performed using less invasive procedures, generating less anatomopathological material for analysis. That circumstances increase the need of careful handling of the biopsy specimen material. Moreover, an aggravating situation is the demand for the identification of a growing number of molecular targets for treatment[7].

A phase 2 trial, keynote-021, has compared pemetrexed with carboplatin with or without pembrolizumab in metastatic non-squamous NSCLC. The overall rate response was better (56.7%) in the group with pembrolizumab after 23.9 mo when compared with patients that did not receive this drug (30.2%), as well as progression-free survival (PFS), with a hazard ratio (HR) = 0.53. The HR for overall survival (OS) was 0.56 in favor of pembrolizumab[7]. After this trial, there were many trials analyzing the effectiveness of immunotherapy in NSCLC. In patients with the expression of PD-L1 < 50%, some trials tried to have a north in treatment.

The keynote-189, a phase III trial that included unselected patients for PD-L1, also evaluated the above-mentioned treatment. This trial showed that the immunotherapy with chemotherapy *vs* chemotherapy alone presents a significant difference in OS

within 12 mo, 69.2% *vs* 49.5% respectively. Adverse events (AEs) occurred with grade 3 or higher in 67.2% and 65.8%. It is interesting to notice that acute kidney injury grade 3 or higher was more present in pembrolizumab group (5.2% *vs* 0.5%). Referring to immune-mediated effects the group with immunotherapy had 22.7% of grade 3 or higher *vs* 11.9% in the group that lacked pembrolizumab. It is important to note three deaths by pneumonitis (immune-mediated side effect) in that group. Moreover, the correlation between expression of PD-L1 and time of progression or death was also reaffirmed[8].

As an alternative treatment for the patient with non-squamous NSCLC, there are combinations with atezolizumab with bevacizumab, carboplatin and paclitaxel (ABCP). This combination was tested in IMpower 150 trial, in which patients were randomized for the combination above or bevacizumab, carboplatin and paclitaxel (BCP) or atezolizumab, carboplatin and paclitaxel (ACP). After the phase of induction they received bevacizumab or atezolizumab or both in combination until progression or unmanageable toxic effects. From 1202 patients enrolled in three groups, almost half were PD-L1 negative. It is important to be highlighted that patients with drive mutation were included in this study because the specific therapy was not available in their countries. PFS was significantly longer in the group of atezolizumab, 8.3 mo *vs* 6.8 mo (BCP). The same occurred with OS: 19.2 mo *vs* 14.7 mo. The median duration of response was also longer in immunotherapy group (ABCP and ACP), 9.0 mo *vs* 5.7 mo (BCP). Immune-mediated AEs grade 1 and 2 were present in 77.4% of patients with atezolizumab, but no grade 5 side-effect was observed. Deaths by AEs were similar in each group[9].

In 2019, another trial was published with a subgroup of patients from IMpower 150 that were affected by liver metastasis at presentation. Improved median OS was observed in favor of combination of anti-VEGF with immunotherapy group *vs* bevacizumab group, 13.3 mo *vs* 9.4 mo respectively. Importantly, the OS didn't differ when groups ACP *vs* BCP were evaluated. However, the groups ABCP and ACP were not compared[10].

Another trial that validated atezolizumab was the IMpower 130. This trial compared a combination of chemotherapy with atezolizumab *vs* chemotherapy alone. The scheme of chemotherapy was carboplatin with nab-paclitaxel. The median OS and PFS was significantly longer for immunotherapy group, 18.6 mo *vs* 13.9 mo and 7.0 mo *vs* 5.5 mo[11].

Nivolumab and Ipilimumab are an option to treat in first-line non-squamous and squamous NSCLC. This therapy was evaluated by a study conducted by Hellmann *et al*[12], 2019. In such a study, almost 70% of included patients had non-squamous NSCLC. Patients with PD-L1 > 1% were separated into three groups: Nivolumab alone, nivolumab with ipilimumab or chemotherapy alone. On the other hand, patients with PD-L1 < 1% were randomized for nivolumab plus ipilimumab, nivolumab with chemotherapy or chemotherapy alone. The median OS in PD-L1 > 1% was 17.1 mo in nivolumab and ipilimumab *vs* 14.9 mo in the chemotherapy group, while in PD-L1 < 1% patients, that measure was of 17.2 mo in ipilimumab and nivolumab *vs* 12.2 mo in the chemotherapy group. With regards to AEs we could observe similarity between groups, 32.8% with ipilimumab and nivolumab *vs* 36% in chemotherapy group. In immunotherapy combination, the most common side effects were skin reactions and endocrine events. Treatment-related deaths were too similar, 8 patients in immunotherapy combination *vs* 6 in chemotherapy group[12].

A different trial evaluated pembrolizumab *vs* chemotherapy in patients with more than 50% of PD-L1 expression. Almost 30% of the patients had squamous NSCLC. The median OS was 30 mo in pembrolizumab group *vs* 14.2 mo in the chemotherapy group. AEs grade 3 to 5, incidences of serious treatment-related effects and treatment discontinuation were similar in both groups. Immune-mediated reactions occurred in 33.8% in pembrolizumab groups and in 5.3% in the chemotherapy group. There were 2 deaths in the immunotherapy group *vs* 3 in the chemotherapy one[13].

In keynote 042 the included patients had squamous (38%) and non-squamous (62%) NSCLC. The population was divided into stratum of PD-L1 expression, ≥ 50%, ≥ 20% and ≥ 1%. In patients with PD-L1 ≥ 50% the OS of pembrolizumab group was 20 mo *vs* 12.2 mo in patients that did not receive the therapy. The same was observed in PD-L1 ≥ 20%, with 17.7 mo *vs* 13 mo, and in PD-L1 ≥ 1%, 16.7 mo *vs* 12.1 mo. Adverse immune-mediated events and infusion reactions, grade 3 or worse, were higher in pembrolizumab group, 8% *vs* 1%, and, in all grade side effects, 28% *vs* 7%. The most common grade 3 or worse in immune-mediated adverse effects in the immunotherapy group were pneumonitis, severe skin reactions and hepatitis[14].

Since we have no trials comparing pembrolizumab *vs* chemotherapy and pembrolizumab, Zhou *et al*[15], 2019 published an indirect comparison meta-analysis trying to solve this problem. It was evaluated the efficacy of pembrolizumab plus chemotherapy *vs* pembrolizumab alone (pem) in first-line treatment of NSCLC and

PD-L1 ≥ 50%. Comparisons were made with 2 blocks of two groups. Arm A with pembrolizumab plus chemotherapy *vs* arm C with chemotherapy and arm B with pembrolizumab *vs* arm C with chemotherapy. Five trials were selected, keynote 021, 189, 407, 024 and 042. In direct meta-analysis OS was better for the use of pembrolizumab with chemotherapy or alone *vs* chemotherapy alone, HR = 0.51 and 0.67 respectively. In indirect meta-analysis, however, the result was not observed, HR = 0.76, confidence interval: 0.51–1.14[15].

Nivolumab as first-line therapy was not successful. A trial published in 2017 compared nivolumab alone *vs* chemotherapy alone. The median PFS was 4.2 mo with nivolumab *vs* 5.9 mo with chemotherapy and median OS was 14.4 *vs* 13.2 mo respectively. AEs of grade 3 or 4 were lower in nivolumab *vs* chemotherapy group, 18% *vs* 51%. The most immune-mediated AEs were skin-related[16].

Regarding Durvalumab, in an abstract published in 2018, 1118 patients were randomized for durvalumab *vs* chemotherapy and durvalumab plus Tremelimumab *vs* chemotherapy in NSCLC. However, no statistical significance in OS and PFS was observed between groups[17]. Two abstracts show better OS when patients had PD-L1 ≥ 25% and used statistical methods to evaluate the effect of subsequent immunotherapy, HR = 0.66 in favor of durvalumab[18,19]. Another abstract was published analyzing patients with high tumor mutational burden. In this group of patients median OS was better in durvalumab alone and associated with tremelimumab *vs* chemotherapy, HR = 0.77 and 0.49 respectively[20].

IMMUNOTHERAPY AFTER AT LEAST ONE LINE IN METASTATIC NON-SQUAMOUS NSCLC

Nivolumab was compared with docetaxel as second-line after platinum-based doublet chemotherapy. The median OS was longer in the immunotherapy group, 12.2 mo *vs* 9.4 mo. AEs grade 3 and 4 were lower in nivolumab group, 10% *vs* 54%. The most common AEs in nivolumab group were fatigue, nausea, decreased appetite, and asthenia. In immunotherapy treatment, pneumonitis (3% *vs* < 1%) and hypothyroidism (7% *vs* 0%) were more frequent. There were 2 deaths, one in each group (by encephalitis in the nivolumab group and by febrile neutropenia in the docetaxel group)[21]. In an update of this study, the median OS in two years was 29% in nivolumab group *vs* 16% in docetaxel group. The duration of response was longer in the nivolumab group, with 34% of the responders having a response even within two years. In patients in whom immune-modulating medications were administered to manage treatment-related AEs, nearly all AEs resolved[22]. In patients pools of checkmate 017 (squamous NSCLC) and 057 (non-squamous NSCLC), mean treatment duration of patients treated with nivolumab and docetaxel was 8.3 mo and 3.1 mo, respectively[23]. It was suggested that nivolumab provides health-related quality of life advantages compared with docetaxel, in addition to longer OS, in previously treated patients with advanced non-squamous or squamous NSCLC[24].

Atezolizumab is another option to treat patients after at least one line of treatment. A trial with non-squamous and squamous NSCLC evaluated patients to receive atezolizumab or docetaxel. It had excluded patients with previous immunotherapy or docetaxel use. The median OS was better in immunotherapy *vs* docetaxel group, 13.8 mo *vs* 9.6 mo. Patients with PD-L1 expression ≥ 50% had the greatest benefit from atezolizumab, median OS 20.5 mo *vs* 8.9 mo. AEs grade 3 to 4 were 37% in atezolizumab group *vs* 54% in docetaxel group. The most common AEs were fatigue, nausea, decreased appetite and asthenia in the immunotherapy group. Musculoskeletal pain and pruritus, immune-mediated AEs, were more common in this group. Complementarily, pneumonitis was observed in 6 patients (grade 3), hepatitis was present in 2 patients (grade 3) and colitis also affected 2 patients (grade 2)[25]. The 24-mo landmark OS rate (30.9% *vs* 21.1%) and HR (0.75) were better for atezolizumab group. In this trial, the most common immune-related AEs were rash (16.9%) and hepatitis (12.6%). Hypothyroidism (4.8%) and pneumonitis (2.3%) were also present[26].

Pembrolizumab after first-line was tested in 2016. In keynote 010, NSCLC patients were randomized in pembrolizumab (high or low dose) and docetaxel groups, of which almost 20% had squamous NSCLC and about 70% had non-squamous NSCLC. Moreover, all of them presented PD-L1 expression ≥ 1%. The median OS was 12.7 mo (high dose) and 10.4 mo (low dose) *vs* 8.5 mo (docetaxel)[27]. An update with patients that completed 2 years of treatment and patients who received a second course of pembrolizumab was published. The OS remained longer for immunotherapy treatment with HR = 0.53 and 0.69 for PD-L1 expression ≥ 50% and ≥1%, respectively[28]. The health-related quality of life was evaluated in keynote 010 and it

was better for patients in immunotherapy group[29]. The most common AEs were hypothyroidism, hyperthyroidism, and pneumonitis. Deaths occurred in five patients of each group, pneumonitis was the cause of 3 deaths in pembrolizumab group[27].

In JAVELIN lung 200 trial, avelumab was evaluated *vs* docetaxel after at least one line of treatment. All patients had expression of PD-L1. The median OS did not differ significantly between both groups, 11.4 mo *vs* 10.3 mo[30].

IMMUNOTHERAPY IN METASTATIC SQUAMOUS NSCLC

There were fewer trials with only squamous NSCLC patients evaluating immunotherapy since most studies mainly evaluated patients with non-squamous NSCLC. The checkmate 227, keynote 042 and keynote 024 cited above had 30%, 38%, and 29% of squamous NSCLC patients, respectively[12,13,14].

The keynote 407 evaluated pembrolizumab only in patients with squamous NSCLC with PD-L1 expression ≥ 1%, as first-line treatment. The individuals were randomized for pembrolizumab *vs* placebo with carboplatin in combination with paclitaxel or nab-paclitaxel for 4 cycles. Pembrolizumab was maintained up for 35 cycles. The median OS was better in immunotherapy group, 15.9 mo *vs* 11.3 mo. Immune-mediated AEs and infusion reactions occurred in 28.8% in pembrolizumab group *vs* 8.6% in placebo group. Grade 3 or higher AEs were present in pembrolizumab group (10.8%) and placebo group (3.2%). There was one death in each group, both by pneumonitis. In pembrolizumab group, the most common grade 3 to 5 immune-mediated AEs were pneumonitis (2.5%), colitis (2.2%), and hepatitis (1.8%), while the other ones accounted less than 1%[31].

Another trial that evaluated only squamous NSCLC was IMpower 131. Patients were randomized for atezolizumab *vs* placebo, both associated with chemotherapy. The median PFS was 6.3 mo *vs* 5.6 mo, better for immunotherapy[32]. An update of that study published and presented by Jotte *et al*[33], 2019, at the International Association for the Study of Lung Cancer 2019 World Conference on Lung Cancer, concluded that median OS was 14.2 mo for atezolizumab *vs* 13.5 mo for placebo. In high expression PD-L1 patients, the median OS was 23.4 mo *vs* 10.2 mo, better for atezolizumab group. It is important to emphasize that the above-mentioned data were published only in abstracts[33].

There is a trial published in 2015 that compared nivolumab *vs* chemotherapy after at least one prior therapy in metastatic setting in patients with squamous NSCLC. The median OS was better for the immunotherapy group, 9.2 mo *vs* 6 mo. All patients had an expression of PD-L1. The major cause of discontinuation of treatment in nivolumab group was pneumonitis (2%), and no death was related in this group[5].

IMMUNOTHERAPY IN ADJUVANT SCENARIO ON NSCLC

Durvalumab was compared to placebo in stage III NSCLC patients after unsuccessful chemoradiotherapy. The 24-mo OS rate was better in the immunotherapy group (66.3% *vs* 55.6%). The median time to death or distant metastasis was 28.3 mo *vs* 16.2 mo also better in the durvalumab group. The most frequent AE leading to the discontinuation of treatment was pneumonitis (4.8% in durvalumab group *vs* 2.6% in placebo group). Death due to AEs occurred in durvalumab (4.4%) and placebo group (6.4%)[34]. After that trial, the idea of immunotherapy for adjuvant setting was rekindled, and, nowadays, have been evaluated by an amount of current studies[35] (Table 1).

IMMUNOTHERAPY IN SMALL CELL LUNG CANCER

SCLC patients present lower survival rates when compared to NSCLC. Immunotherapy presents various limitations in SCLC, but advances in this field have been achieved.

There is no data on the use of immunotherapy as primary or adjuvant therapy in limited-stage SCLC. However, atezolizumab has been incorporated for extensive-stage disease. A phase III trial randomized patients with extensive-stage SCLC for carboplatin and etoposide with atezolizumab or placebo for four cycles followed by maintenance phase. The median OS was 12.3 mo (atezolizumab) *vs* 10.3 mo (placebo). It is interesting to note that the patients were not tested for PD-L1 due to the non-standardization of samples obtained and because a low PD-L1 prevalence in tumor cells were expected. Moreover, no evidence of correlation between PD-L1 expression

Table 1 Ongoing trials on lung cancer immunotherapy

Trial	Aims	Registry number[1]
ANVIL	To compare nivolumab *vs* placebo in stage IB-IIIA squamous NSCLC	NCT02595944
PEARLS	To evaluate pembrolizumab *vs* placebo in stage IB, II and IIIA NSCLC	NCT02504372
IMpower 010	To randomize stage IB-IIIA NSCLC patients to receive atezolizumab following adjuvant platinum-based chemotherapy or best supportive care	NCT02486718
BR31	To assess durvalumab *vs* placebo in completely resected tumors	NCT02273375

[1]ClinicalTrials.gov registry number. NSCLC: Non-small cell lung cancer.

and atezolizumab activity have been described. The HR was 0.7 in benefit for atezolizumab. AEs of any grade were similar in both groups. In the atezolizumab group the most common grade 3 and 4 AEs were neutropenia, anemia and decreased neutrophil count. There were three deaths in each group. In atezolizumab group, they occured due to pneumonia in one patient and neutropenia in another patient. Moreover, one death verified in this group presented unspecified cause. Regarding placebo group, deaths were due to pneumonia, septic shock and cardiopulmonary failure. Immune-mediated AEs, mainly hypothyroidism and rash, occurred in 39.9% *vs* 24.5% in atezolizumab and placebo group, respectively. Most grade 3 and 4 immune-mediated AEs in atezolizumab group were rash (2%), infusion-related reaction (2%), hepatitis (1.5%), and colitis (1%)[36].

In the treatment of patients suffering relapses of 6 mo or fewer, some immunotherapy options have been tried, but the treatment standardization is being sought by some trials. In this framework, one possibility is the combination of ipilimumab and nivolumab. There is a phase 1/2 trial that tested nivolumab alone or nivolumab with ipilimumab for patients with limited or extensive-stage diseases after progression with at least one platinum-based chemotherapy regimen. Due to the same reasons reported by the above-mentioned trial, PD-L1 expression was not tested, and this parameter was assessed only retrospectively. There were four groups with different doses of nivolumab and ipilimumab. The objective response was 23% in nivolumab 1 mg/kg plus ipilimumab 3 mg/kg group *vs* 10% in nivolumab 3 mg/kg group *vs* 19% in nivolumab 3 mg/kg plus ipilimumab 1 mg/kg group *vs* 33% in nivolumab 1 mg/kg plus ipilimumab 1 mg/kg group (just 3 patients in this group). Grade 3 and 4 AEs occurred in 30%, 13%, 19%, 0% patients, respectively, and the most common of them were increased lipase and diarrhoea[37].

An update of Checkmate 032 was published as abstract in 2017. In which, the randomization was to nivolumab or nivolumab 1 mg/kg plus ipilimumab 3 mg/kg. The median disease control rate was 36% *vs* 49%, respectively[38].

Pembrolizumab is another possibility in SCLC immunotherapy. That drug was tested in phase 2 trial with advanced SCLC with one or two previous treatment. All patients were tested for PD-L1 expression and it was positive if ≥ 1%. The objective response rate was 35.7% in positive PD-L1 expression and 6% in negative PD-L1 expression. The median OS was 14.6 mo in PD-L1 positive group and 7.7 mo in PD-L1 negative group[39].

There are few studies published for immunotherapy in SCLC, but there are almost 30 open trials with immunotherapy in different lung cancer settings[40]. Figure 1 summarizes the safety level for an indication of immunotherapeutic drugs in the treatment of lung cancer.

CONCLUSION

As a conclusion, immunotherapy is considered the new standard in advanced and metastatic NSCLC, with or without chemotherapy. Moreover, it is evident that adequate immunohistochemistry is essential in NSCLC approach since it strongly correlates with treatment response. The benefit of immunotherapy was documented in patients with different sites of metastasis, chemotherapy combination, and expression of PD-L1, although OS in patients with PD-L1 expression ≥ 50% was higher. It is important to be highlighted that promising results have been achieved in

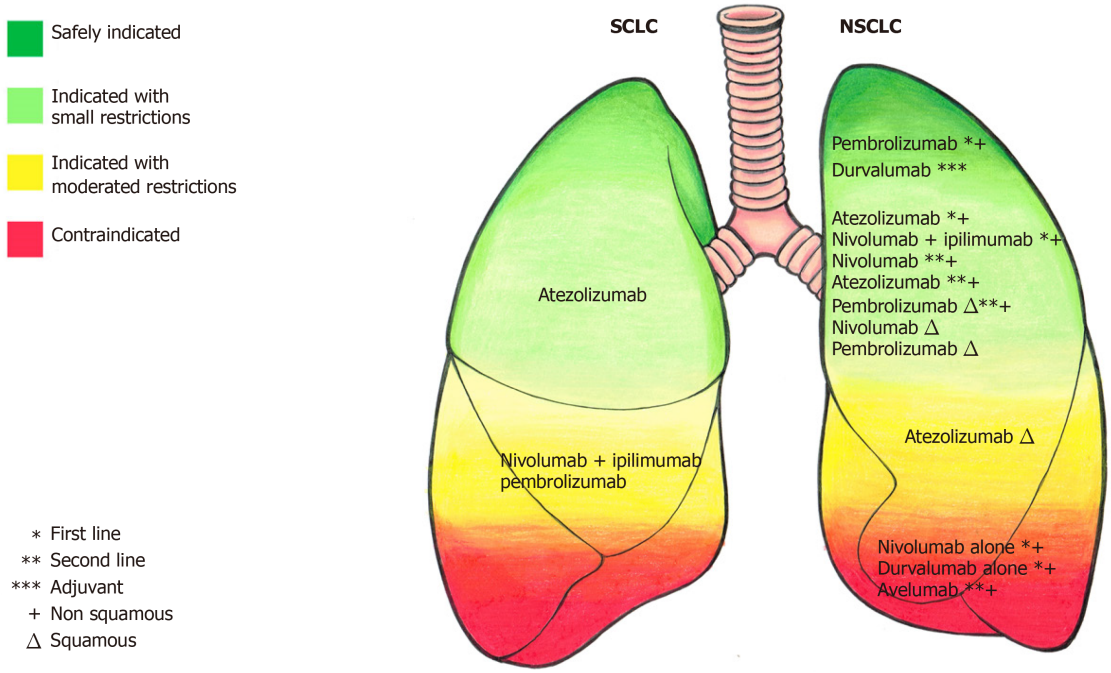

Figure 1 Safety level for indication of immunotherapeutic drugs in the treatment of lung cancer. SCLC: Small-cell lung cancer; NSCLC: Non-small-cell lung cancer.

both squamous and non-squamous NSCLC, as well as in SCLC. Furthermore, either in first-line or after at least one line, immunotherapy has presented good effects. However, almost all the trials that tested immunotherapy showed immune-mediated AEs and even deaths related to this. In addition, when two immunotherapies are combined, the immune-mediated side effects tend to be worse. The most commonly observed immune-mediated AEs were pneumonitis, hepatitis and skin reactions. In a near future, it is expected that new treatment schemes involving immunotherapy and its combinations will be established. Even now, there are many doubts on what are the optimal doses and the adequate duration for immunotherapies. Finally, the expansion of the knowledge about the use of this therapeutic modality as adjuvant treatment, and new studies on the immune-mediated adverse effects due to these treatments will improve their application in clinical practice.

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REVIEW

CITED2 and the modulation of the hypoxic response in cancer

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Abstract

CITED2 (CBP/p300-interacting transactivator with Glu/Asp-rich C-terminal domain, 2) is a ubiquitously expressed protein exhibiting a high affinity for the CH1 domain of the transcriptional co-activators CBP/p300, for which it competes with hypoxia-inducible factors (HIFs). CITED2 is particularly efficient in the inhibition of HIF-1α-dependent transcription in different contexts, ranging from organ development and metabolic homeostasis to tissue regeneration and immunity, being also potentially involved in various other physiological processes. In addition, CITED2 plays an important role in inhibiting HIF in some diseases, including kidney and heart diseases and type 2-diabetes. In the particular case of cancer, CITED2 either functions by promoting or suppressing cancer development depending on the context and type of tumors. For instance, CITED2 overexpression promotes breast and prostate cancers, as well as acute myeloid leukemia, while its expression is downregulated to sustain colorectal cancer and hepatocellular carcinoma. In addition, the role of CITED2 in the maintenance of cancer stem cells reveals its potential as a target in non-small cell lung carcinoma and acute myeloid leukemia, for example. But besides the wide body of evidence linking both CITED2 and HIF signaling to carcinogenesis, little data is available regarding CITED2 role as a negative regulator of HIF-1α specifically in cancer. Therefore, comprehensive studies exploring further the interactions of these two important mediators in cancer-specific models are sorely needed and this can potentially lead to the development of novel targeted therapies.

Key words: Cancer; Cancer stem cell; CBP/p300; CITED2; Hypoxia-inducible factors 1α;

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Hypoxia; Leukemia; Tumor

Core tip: Hypoxia is a common feature of many cancers. In response to hypoxia, hypoxia-inducible factor 1 (HIF-1) is stabilized and activates downstream target-genes participating in crucial aspects of cancer biology, such as angiogenesis, cell survival, glucose metabolism and invasion. CITED2 is a negative regulator of HIF with demonstrated roles in various types of cancer. Therefore, CITED2 can potentially modulate HIF effects in cancer and constitute a novel target for therapies. Herein, we compile the roles reported for CITED2 in health and disease, namely through the modulation of HIF activity. We also discuss the various context-dependent roles for CITED2 in cancer.

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INTRODUCTION

CITED2, formerly known as melanocyte-specific gene related gene 1 and p35srj[1-4], is a member of the CBP/p300-interacting transactivator with Glu/Asp-rich C-terminal domain family of transcriptional modulators that includes also CITED1, CITED3 and CITED4. These proteins, characterized by a conserved CBP/p300-interacting domain located at the C-terminal domain, termed CR2 for conserved region 2 (Figure 1A), are only conserved in vertebrates and contribute to several aspects of embryogenesis and/or to normal organ function in adult organisms[1,3,5-7]. CBP and p300 are homologous nuclear phosphoproteins, ubiquitously expressed, that contain amongst others, three highly conserved cysteine–histidine-rich domains (CH1, -2, and -3) and function as transcriptional coactivators[8,9]. Although CITED proteins lack a DNA-binding domain, they usually localize in the nucleus where they function as transcriptional modulators[10,11]. From all CITED members, CITED2 has been the most studied, due to its pivotal roles in many different biological processes[5,6,12-14]. The CR2 of CITED2 is composed of 32 amino acids (residues 224 to 255) that contain a potent transactivation domain (TAD), which is responsible for physically interacting with the CH1 domain of CBP/p300, also known as TAZ1[3] (Figure 1A). The CH1 domain of p300, which has an extremely high affinity for CITED2, also binds hypoxia-inducible factors (HIF)-1α[15] and other transcription factors such as the NFκB p65 subunit[16], p53, and MDM2[17]. Due to its interaction with CBP/p300, CITED2 was reported to act both as a positive and a negative regulator of transcription. This function appears to depend on whether CITED2 enables the interaction between CBP/p300 and other transcription factors, or whether it prevents such interactions. CITED2 has been reported to be a transcriptional coactivator of TFAP2, SMAD2/3 (mediating TGFβ signaling), peroxisome proliferator-activated receptors, ISL1, amongst other factors[6,18-20]. Conversely, it acts as a transcriptional repressor through interfering with the binding of transcription factors, such as HIF-1α and STAT2, with CBP/p300[21]. CITED2 is ubiquitously expressed and its expression is modulated by many biological stimuli including lipopolysaccharide and cytokines, such as interleukin 9 and interferon gamma, in different cell types[2]. Most notably, CITED2 is highly inducible by the HIFs under low oxygen/hypoxic conditions[3].

CITED2 AND THE MODULATION OF THE HYPOXIC RESPONSE

The cellular response to hypoxia is critical for cell survival and is strictly regulated to allow cells to adjust their needs during altered oxygen levels. HIFs play a central role in systemic and cellular adaptation to decreased oxygen levels. These transcription factors are heterodimeric proteins consisting of a hypoxia-inducible HIFα subunit, and a constitutively expressed HIFβ subunit, also known as Aryl Hydrocarbon

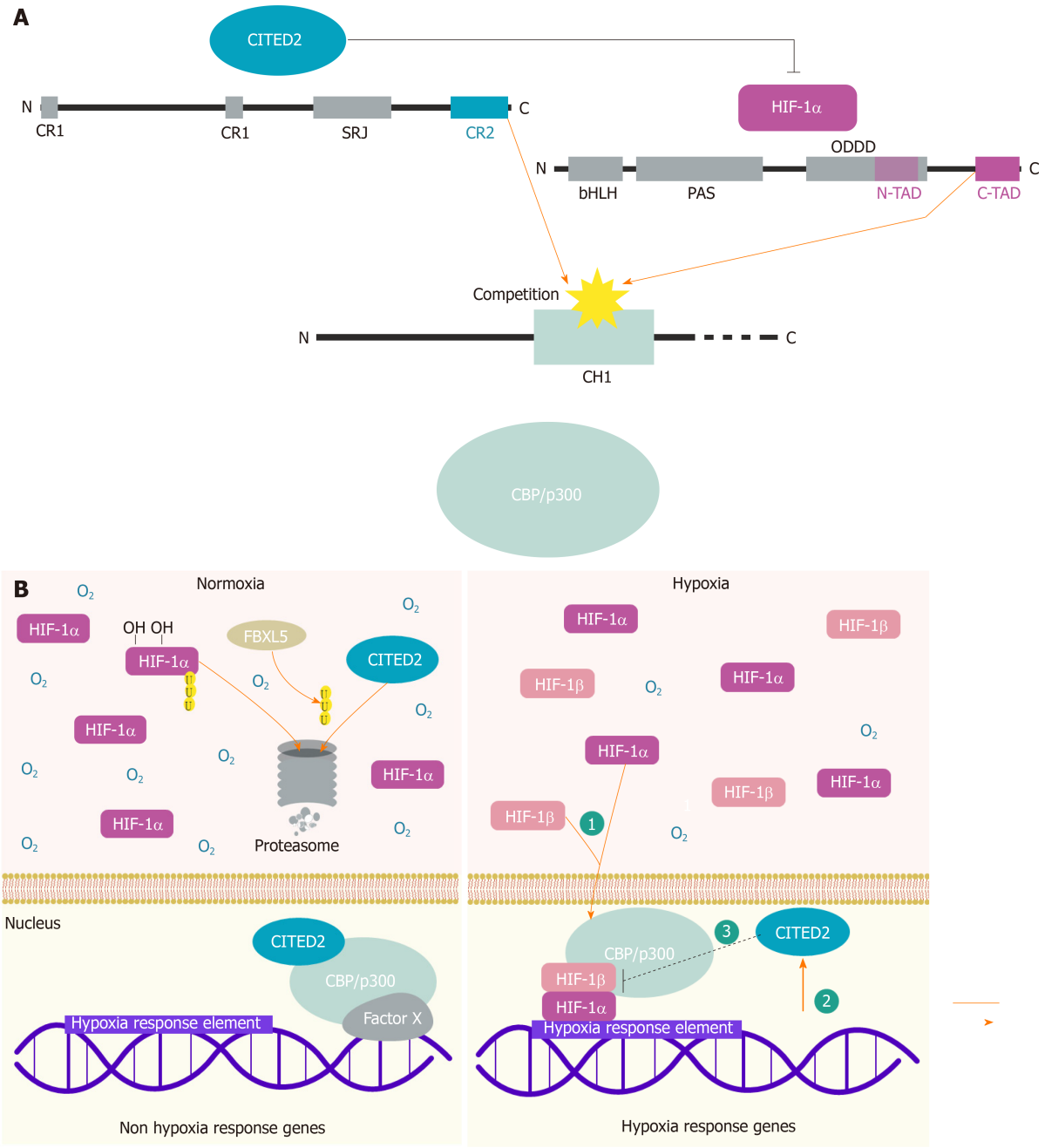

Figure 1 Structure of CITED2 and its interplay with hypoxia-inducible factors to modulate the response to hypoxia. A: Schematic representation of CITED2, hypoxia-inducible factor (HIF)-1α and CBP/p300. The conserved regions amongst CITED family members, and the serine-glycine rich junction domain unique to CITED2 are indicated. Conserved regions 2 is the domain that characterizes the CITED proteins that contains a potent transactivation domain (TAD), which is responsible for the physical interaction with the domain CH1 of CBP/p300, also represented. HIF-1α has two TADs, namely, a N-terminal TAD and a C-terminal TAD, which are responsible for the transcriptional activity under hypoxia. To this end, HIF-1α binds to the CH1 region of p300, but CITED2 was shown to compete out HIF-1α for the binding to the same region, and to interfere with hypoxia-driven transcription; B: Model for CITED2, HIF-1α and CBP/p300 in hypoxia-responsive gene regulation. In normal levels of oxygen (normoxia), HIF-1α is hydroxylated at two proline residues within its oxygen-dependent degradation domain, which marks HIF-1α for proteasome degradation. In normoxia, CITED2 binds to CBP/p300 in the nucleus regulating the expression of target-gene. In addition, part of CITED2 proteins is ubiquitinated by FBXL5 and degraded by the proteasome. In hypoxia, HIF-1α is no longer hydroxylated, which prevents its degradation by the proteasome. Consequently, HIF-1α translocate and accumulates in the nucleus to associate with the HIFβ subunit to bind to specific Hypoxia-Response Elements in hypoxia-regulated genes. CITED2 competes with HIF-1α for the binding to the CH1 region of CBP/p300 and interferes with hypoxia-driven transcription. CR: Conserved regions; SRJ: Serine-glycine rich junction; TAD: Transactivation domain; HIF: Hypoxia-inducible factor; NAD: N-terminal TAD; CAD: C-terminal TAD.

Receptor Nuclear Translocator or ARNT[22,23]. Three oxygen-sensitive HIF subunits have been identified to date, HIF-1α, HIF-2α, and HIF-3α, and all of them dimerize with HIF-1β, originating HIF-1, HIF-2, and HIF-3 heterodimers, respectively[24]. HIF-1α, HIF-2α and HIF-3α have a high degree of sequence homology but are significantly distinct in their functions. For instance, the hypoxic response may be exclusively controlled by one or the other oxygen-sensitive HIF isoforms in different contexts[25]. Since several studies have reported a role for CITED2 in hypoxia, particularly through

the inhibition of the HIF-1α, we will focus the review on this isoform.

HIF-1α cellular levels are essentially regulated through alterations in protein stability. In normal oxygen levels (normoxia), HIF-1α (and also HIF-2α) is constantly expressed, but rapidly degraded through the ubiquitin-proteasome pathway, in an oxygen-dependent manner[26-29]. HIF-1α contains an oxygen-dependent degradation domain (ODDD) which is hydroxylated at two proline residues by prolyl-4-hydroxylase domain proteins (PHDs), known as oxygen sensors[30,31]. The prolyl-hydroxylation allows the interaction of HIF-1α with the von Hippel-Lindau (VHL) protein, which is the substrate recognition component of an E3 ubiquitin ligase complex, leading to HIF-1α degradation by the proteasome[32-36] (Figure 1B). HIF-1α has two TADs, namely, a N-terminal TAD (NAD) located within the ODDD and a C-terminal TAD (CAD), which are responsible for the transcriptional activity of HIF-1α (and HIF-2α) under hypoxia (Figure 1A). In addition to the ODDD regulation, which prevents the stability of HIF proteins, a second oxygen-dependent mechanism is responsible for the inhibition of the HIF-1α transcriptional activity. Indeed, an asparagine residue located in the HIF-1α CAD (and HIF-2α CAD), is the target for hydroxylation by an oxygen-dependent hydroxylase named factor inhibiting HIF or FIH-1[21,37]. The hydroxylation of this asparagine residue interferes with the recruitment of the CBP/p300 in normoxia[38,39]. Therefore, molecular mechanisms involving the hydroxylation of ODDD and CAD synergize to neutralize HIF-1α (and HIF-2α) transcriptional activity in normoxia.

In hypoxia, the prolyl-4-hydroxylase domain proteins lose their ability to hydroxylate HIF-1α, which prevents its interaction with VHL and proteasomal degradation. Consequently, HIF-1α translocates and accumulates in the nucleus, and associates with the HIFβ subunit to form HIF-1 and to bind to specific hypoxia-response elements in hypoxia-regulated genes (Figure 2B). Thus, HIF-1 is responsible for transcriptional programs that promote erythropoiesis, glycolysis, and angiogenesis, between other processes[22,40,41]. It was previously demonstrated that HIF TADs play different roles in hypoxia responses by activating distinct subsets of genes[42,43]. Nevertheless, the expression of hypoxia-responsive genes is only efficient when HIF-1 recruits CBP/p300[15].

CITED2 was shown to compete with HIF-1α for the binding to the CH1 region of CBP/p300, and to interfere with hypoxia-driven transcription[3] (Figure 1A). Structural studies have shown that both HIF-1α and CITED2 transactivation domains interact with the CH1 domain of CBP/p300 through helical motifs that flank a conserved LP(Q/E)L sequence to achieve high affinity binding, displacing each other in a feedback loop during the hypoxic response[10,43-45]. More specifically, Berlow *et al*[45] have shown that CITED2 is able to displace HIF-1α by forming a transient ternary complex with TAZ1/CH1 and HIF-1α, competing for a shared binding site through its LPEL motif, resulting in a TAZ1 conformational change that increases the rate of HIF-1α dissociation, even at modest concentrations of CITED2[45]. Moreover, CITED2 is itself an hypoxia-stimulated gene through the action of HIF-1α and HIF-2α[3,46]. Thus, CITED2 is thought as a negative feedback regulator of HIF-1α, contributing for the cellular mechanisms that attenuate the hypoxic response.

In addition to reducing the hypoxia-induced activation of HIF CAD-dependent genes, CITED2 also interferes with HIF NAD-dependent gene activation. Indeed, Yoon *et al*[47] showed that NAD interacts with the CH1 and CH3 domains of p300 and these were both required for NAD-dependent transactivation. Interestingly, CITED2 was shown to be able to inactivate NAD by interfering with the NAD-CH1 binding, but not with the NAD-CH3 interaction[47]. Nevertheless, CITED2 is a powerful inhibitor HIF-1α transcriptional activity by blocking the recruitment of CBP/p300 through both NAD and CAD. Remarkably, the same authors found that NAD activation through binding to p300 could be blocked not only by CITED2 but also by the VHL protein, supporting the hypothesis that NAD might be controlled by VHL protein during normoxia and by CITED2 during hypoxia[47]. In this context, we have reported another mechanism supporting a role for CITED2 in HIF-1α NAD inactivation in normoxia. Indeed, we have demonstrated that F-Box and leucine-rich repeat protein 5 (FBXL5) overexpression in cells, results in CITED2 degradation, which enabled the transcriptional activity of HIF-1α through the NAD in normoxia (Figure 1B). In fact, we showed that CITED2 and FBXL5 proteins interact, and FBXL5, which is a substrate adaptor protein part of E3 ubiquitin ligase complexes, triggers a proteasome-dependent degradation of CITED2 and consequently contributes to the modulation of gene expression mostly through HIF-1α NAD[11].

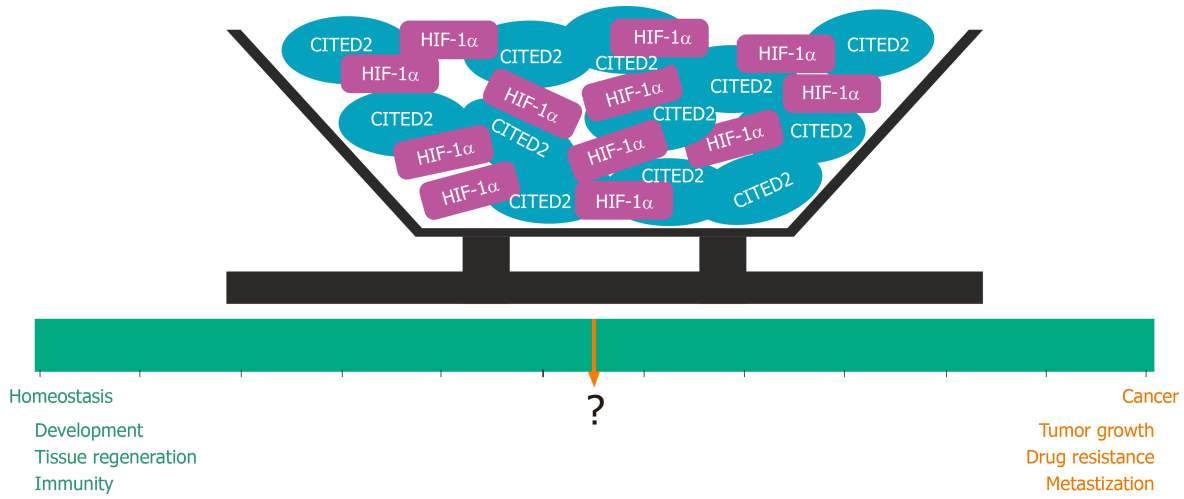

Figure 2 Roles of CITED2 in homeostasis and cancer. CITED2-dependent modulation of hypoxia-inducible factor (HIF) is fine-tuned to control physiological processes such as development, tissue regeneration and immunity. Abrogation of CITED2/HIF-1α homeostasis may lead to the initiation, and/or disease progression depending on the cellular context. Cancer may be one these diseases, in which CITED2 and HIF-1α intervene to control tumor growth, drug resistance and metastization. HIF: Hypoxia-inducible factor.

CITED2 AND HYPOXIA IN PHYSIOLOGICAL PROCESSES

The role of CITED2 in regulating HIF1 activity was shown to be also essential *in vivo*. Indeed, *Cited2* knockout mouse embryos died in utero at around mid to late gestation and displayed multiple developmental anomalies, such as heart malformations, adrenal agenesis and neural tube defects[6]. Interestingly, the hearts of Cited2-null embryos displayed elevated expression of HIF-1α target genes, such as vascular endothelial growth factor (VEGF), glucose transporter 1, and phosphoglycerate kinase 1. These genes were also upregulated in Cited2-depleted mouse embryonic fibroblasts under hypoxic conditions[48]. Interestingly, a normalized expression of HIF-1α target genes and part of the heart defects were rescued upon the heterozygous deletion of an *Hif1a* allele in Cited2-null embryos[48,49]. This indicated that a hyperactivity of HIF-1α in Cited2-null embryos is, at least in part, responsible for the cardiac developmental anomalies observed. Cited2-deficient mouse embryos present also defects in the developing eye and showed an excessive and disorganized hyaloid vasculature with increased VEGF expression[50]. Further experiments showed that deletion of *Hif1a* in the lens could specifically rescue the previous phenotype, supporting the notion that Cited2 is required for the proper hyaloid vascular system regression through negative modulation of HIF-1 signaling during eye development[50]. Cited2 was also reported to modulate HIF-dependent expression of VEGF in the nucleus pulposus of the rat intervertebral disc, most likely contributing for the mechanism by which the cells of the nucleus pulposus survive in a hypoxic environment[51].

Normal hematopoiesis also relies on the correct function of CITED2. Indeed, CITED2 is crucial for the maintenance of the hematopoietic stem cell (HSC) pool in the bone marrow (BM), and its conditional deletion in the hematopoietic system resulted in a dramatic loss of adult HSCs and primitive progenitor cells, ultimately leading to BM failure[52]. In addition, it was reported that Cited2-deficient HSCs presented impaired quiescence and reconstitution capacity, which could be partially rescued by additional deletion of HIF-1α[53]. Therefore, Cited2 is able to regulate HSC quiescence through a HIF-1-dependent mechanism, although other HIF-1-independent mechanism(s) may be also involved in this context[53]. In addition, CITED2 was reported to be involved in immunity, more specifically by repressing macrophage-mediated inflammation. Thus, Cited2 deficiency may enhance proinflammatory gene expression through stabilization of HIF-1α in macrophages. Furthermore, the inhibition of HIF-1α in Cited2-deficient macrophages completely reversed the elevated proinflammatory cytokine/chemokine gene expression[54]. Thus, the repressive CITED2 action on HIF-1α activity is crucial for many aspects of mouse and rat development, as well as adult homeostasis.

CITED2 AND HYPOXIA IN DISEASE

CITED2 was shown to be involved in the regulation of the hypoxic response in the context of diseases (Figure 2). Using animal models of chronic kidney disease and heart failure, Tanaka *et al*[55] have implicated CITED2 in the negative regulation of HIF-target genes, and consequently in suppressing the hypoxic response. In this context, the accumulation of indoxyl sulfate (an uremic toxin) in the systemic circulation due to a reduced renal clearance, resulting in renal and cardiovascular dysfunction[56], was due to CITED2 stabilization and consequent HIF-1α inhibition[55]. Also, in a type 2 diabetes animal model, Wang *et al*[57] have shown that, although insulin is able to downregulate CITED2 in endothelial cells, the vascular insulin resistance characteristic of this disease contributes to the upregulation of CITED2, which impairs HIF signaling and, consequently aborts angiogenesis[57].

In cancer, HIF-1 activates the transcription of genes that are involved in crucial aspects of cancer progression, including angiogenesis, cell survival, glucose metabolism, invasion and cell self-renewal[58,59]. In several types of cancer, intratumoral hypoxia and genetic alterations were reported to lead to HIF-1α overexpression, which is associated with increased patient mortality. Therefore, the inhibition of HIF-1 activity is regarded as a promising therapeutic approach[22,58]. Importantly, the intricate HIF pathway also initiates anti-tumorigenic mechanisms that lead to cell cycle arrest or cell death[60,61], illustrating the need for a stringent control of the hypoxia response. Thus, HIF feedback regulators help to adjust and adapt HIF-activated responses to the fluctuating oxygen concentrations within tumors and to restrict the tumor-suppressing components of the HIF pathway. Therefore, given their role in cancer biology, HIF feedback regulators such as CITED2 may represent attractive targets for cancer therapy[62].

CITED2 AND HYPOXIA IN CANCER

The potential involvement of CITED2 in cancer was unveiled since it was demonstrated to be a transforming gene in Rat1 cells. Indeed, when overexpressed, Cited2 induced loss of cell contact inhibition, anchorage-independent growth, and tumor formation in nude mice[2]. Since this first publication describing CITED2, the dysregulation of its expression has been widely associated with aggressiveness and prognosis of several cancers including, among others, breast, colon, prostate and gastric cancers, as well as acute myeloid leukemia.

CITED2 in breast cancer

Breast cancer (BC) is the most frequent cancer affecting women worldwide, and the second leading cause of cancer-related deaths in women[63]. Studies using both animal models and human BC primary samples showed that CITED2 expression was elevated in primary tumors and metastasis, when compared to normal mammary epithelium[64,65]. However, the reports on CITED2 function and prognostic in BC are discrepant. Indeed, high CITED2 mRNA levels were associated with a clinical benefit in tamoxifen-treated BC and a prolonged metastasis-free survival in patients who had not received adjuvant systemic therapy[66]. More recent studies, showed that high CITED2 levels in primary tumors, when compared to normal mammary epithelium, were inversely correlated with patient survival[64,67]. In addition, high levels of CITED2 expression were shown to significantly increase the proliferation and migration of MCF-7 and SKBR-3 BC cell lines in culture[65]. By studying the possible mechanism involved in tumor growth and metastization, Jayaraman *et al*[68] have shown that the expression of IKKα and other NF-κB targets, with recognized roles in the metastatic process, were significantly decreased in both MDA-MB-231 and MDA-MB-468 cell lines following *CITED2* knockdown. Moreover, the restoration of IKKα expression in CITED2-depleted cells, restored their invasive capacity[68]. In addition, *CITED2* silencing was also associated with reduced primary tumor growth, influencing the tumor vasculature by preventing TGF-β induction of VEGFA *via* CITED2 recruitment to the *VEGFA* promoter[69]. Interestingly, the authors reported that HIF-1α was not involved in this process[69]. This is a surprising observation, since increasing VEGFA expression and tumor vasculature are prime roles usually attributed to HIF-1α and expected to be counteracted by CITED2. Therefore, this report supports the notion that negative regulation of HIF-1α by CITED2 may be cell-type dependent or somehow impaired in breast cancer cells. Alternatively, CITED2 may in this case act as co-activator of other transcription factors, such as SMAD2/3[18], which are mediators of TGF-β signaling pathway, or TFAP2A, showed to cooperate with CITED2 for normal vascularization of the myocardium during heart development[70]. Other studies also reported a reduction in primary tumor growth due to the

attenuation of tumor-associated macrophage (promoting tumor development and progression) recruitment in response to CITED2 depletion in cancer cells and the consequent downregulation of the macrophage chemoattractant CCL20[71]. This suggests that CITED2 promotes tumor-associated macrophage recruitment and infiltration in breast tumors. Metastases are the ultimate cause of death in BC patients and have a special tropism to develop in the bone. Although the molecular and cellular mechanisms behind BC cell homing and colonization of the bone are not fully understood, it was shown that intracardiac injection of CITED2-depleted NT2.5 mammary tumor cells in neu-N immunocompetent mice inhibited the establishment of bone metastasis and osteolysis, suggesting that CITED2 can promote osteotropism in BC[67]. CITED2 was also implicated in the acquisition of resistance to epirubicin and 5-fluorouracil therapies by inhibiting p53 accumulation[65], as well as resistance to tamoxifen, because it is a transcriptional co-activator of the estrogen receptor in breast cancer cells[64].

Altogether, these studies show that CITED2 is overexpressed in BC, contributing to prognosis, invasion and responsiveness to therapy. Moreover, CITED2 was shown to be induced by FOXO3a and to act as a transcriptional co-factor regulating HIF1-induced apoptosis in mouse embryonic fibroblasts and MCF-7 BC cells[72]. Nevertheless, a role for CITED2 in mediating HIF effects in the context of BC was not clearly reported. Interestingly, its family member, CITED4, was found to be expressed in human BC cell lines and to be inversely associated with HIF-1α activity[73]. In fact, CITED4 was shown to be either expressed at low levels in the nucleus or trapped in the cytoplasm during breast tumor progression, implying in both circumstances that CITED4 had lost its ability to inhibit HIF-1α transcriptional activity, allowing the progression of the tumor size, grade and angiogenesis[73].

Cited2 and colorectal cancer

Colorectal cancer (CRC) is the second most deathly cancer worldwide[74]. CRC usually metastasizes to the liver, which is the cause of high mortality rates. In contrast to what was reported for BC, CITED2 depletion in CRC was associated with enhanced cell invasiveness[75]. Bai *et al*[75] have shown increased matrix metalloproteinase 13 (MMP-13) expression following CITED2 knockdown in RKO and SW480 CRC cell lines, suggesting that CITED2 may downregulate MMP-13 expression and limit invasiveness in CRC[75]. Notwithstanding, neutralizing MMP-13 activity with a monoclonal antibody, only slightly reduced the invasive capacity of SW480 cells expressing reduced levels of CITED2, suggesting that other changes in CITED2 knockdown cells also contributed to the altered invasiveness of these cells[75].

These data suggest that downregulation of CITED2 might have an important role in CRC progression but the mechanism mediating these effects is not fully known. Therefore, further studies should be undertaken to assess how CITED2 expression is modulated and whether low levels of CITED2 expression lead to increased HIF-1α transcriptional activity. Also using the SW480 cell line, Rogers *et al*[76] reported that CITED4 gene silencing modulated adherens/tight junction gene expression and reduced cell proliferation, without affecting apoptosis, colony formation, migration, invasion or adhesion[76].

Cited2 and prostate cancer

Prostate cancer (PC) is the most frequent cancer in men. More than half of the patients with PC present a translocation originating a fusion between the androgen-responsive gene TMPRSS2 and ETS (erythroblast transformation-specific)-family transcription factor genes such as ERG (ETS-related gene) and ETV1[77]. It has been speculated that CITED2 might be involved in PC, since its expression can be activated by ELK-1, another ETS family member[46], that is able to recruit androgen receptor to promote PC cell growth[78]. By testing this hypothesis, Shin *et al*[79] found that ERG was specifically upregulated as a consequence of the TMPRSS2–ERG gene fusion in PC cells. Interestingly, this fusion also upregulated CITED2, which was reported to promote post-translational modifications in nucleolin to enhance the metastatic potential of PC cells[79]. The metastatic facilitation occurs through AKT upregulation and a consequent increase of the epithelial–mesenchymal transition and invasion potential[79]. Therefore, CITED2-nucleolin-AKT signaling pathway should be considered as a potential target for therapies aiming to treat PC and prevent metastasis. A role for CITED2 in the regulation of HIF-1α in prostate cancer was not evaluated although *in vitro* studies have previously shown that CITED2 is highly induced by hypoxia in DU145 prostate carcinoma cells[80].

Cited2 and gastric cancer

Gastric cancer (GC) is a silent and slow developing cancer that usually remains undetected until it reaches advanced stages[81]. The studies of Tang *et al*[82] using both

GC cell lines and human primary samples showed that those cells can be categorized based on the CITED2 expression levels. In addition, CITED2 knockdown in cell lines with high CITED2 expression led to a decrease of their proliferation, mitochondrial membrane potential, colony formation, and an induced cell cycle arrest and apoptosis[82]. These results suggest that CITED2 can be considered as a good target for therapy in GC. Additionally, it was reported that GC cells with low expression of CITED2 are chemoresistant to anthracyclines. Interestingly, the pretreatment of GC cells with low expression of CITED2 with LBH589, an HDAC inhibitor, could reactivate the expression of CITED2 and sensitized them to chemotherapeutic drugs[83]. HIF-1α has been shown to be involved in various processes in GC[84], but the impact of CITED2 modulation on its activity remains to be established.

Cited2 and hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide[85]. CITED2 plays an important role in liver development, as indicated by knockouts of *CITED2* in mice, which present fetal liver hypoplasia, due to increased cell apoptosis and disrupted cell-to-cell contact[86]. In HCC cells, CITED2 was identified as a direct activator of PPARγ, which possesses a tumor suppressive activity. CITED2 was significantly downregulated in primary HCCs when compared with their adjacent non-tumor tissues. In addition, CITED2 knockdown in the hepatocyte cell line LO2 and the HCC cell line Hep3B significantly increased cell viability and clonogenicity[87]. This was attributed to an increased cell cycle transition from G1 to S phase, concomitant with the downregulation of the cyclin-dependent kinase inhibitors $p15^{INK4B}$, $p21^{Wat1/Cip1}$, and $p27^{Kip1}$ and the upregulation of cyclin D1 expression[87]. In contrast, overexpression of CITED2 in HepG2 and BEL7404 HCC cell lines significantly suppressed cell growth by increasing $p21^{Wat1/Cip1}$ and $p27^{Kip1}$[87]. MicroRNAs have been reported to be promising biomarkers for HCC[88]. miR-1468, a novel cancer-related MicroRNAs which overexpression was associated with a poor prognosis in HCC patients, was reported to inhibit PPARγ/AKT signaling pathway through direct suppression of CITED2 and Up-frameshift protein 1, consequently promoting cell proliferation[89]. Jiang *et al*[90] also found that miR-182-5p is upregulated in liver cell lines exposed to the human carcinogen trichloroethylen, which in turn inhibits CITED2 and enhances cell proliferation[90]. These data reinforce the antiproliferative effect of CITED2 in HCC. Interestingly, CITED2 was found to be degraded *via* the ubiquitin-proteasome system and thus to be stabilized by proteasome inhibition[91]. Therefore, although HIF-1α can be upregulated by proteasome inhibition as shown in Hep3B cells and HEK293 embryonic kidney cells, its activity is reduced due to CITED2 interference with HIF-1α binding to p300 in hypoxic conditions[91]. This mechanism sheds light on how proteasome inhibitors may inhibit HIF-1α, which can be used as an anticancer therapy. Despite the knowledge of this important role for CITED2 in mediating the paradoxical responses of HIF-1α to proteasome inhibition, the regulation of the hypoxic response by CITED2 and its contribution to the pathogenesis of HCC remains elusive.

Cited2 and acute myeloid leukemia

Acute myeloid leukemia (AML) is a heterogeneous disease in which genetic and epigenetic factors contribute to the abnormal proliferation and differentiation of myeloblasts, generating a clonal population in the bone marrow[92]. An important regulator of hematopoiesis is the PU.1 transcription factor, which is expressed at low levels in hematopoietic stem and progenitor cells[93] and is crucial for hematopoietic stem and progenitor cells maintenance and differentiation of the myeloid lineage[94-98]. In AML, PU.1 is usually dysregulated by mutations, translocations, and alterations in signal transduction, which promotes the accumulation of immature blasts[99,100]. PU.1 negatively regulates the expression of CITED2, by binding to multiple ETS-binding sites in the CITED2 promoter[101]. As shown by conditional knockout studies, Cited2 is essential for HSC maintenance. In contrast, the lack of Cited2 was not so evident in more committed cells[52]. Andersson *et al*[102] have shown that AML cells have high levels of CITED2 expression[102]. In addition, Korthuis *et al*[101] have shown that CITED2 overexpression alone is enough to maintain the primitive $CD34^{+}CD38^{-}$ HSC pool by decreasing apoptosis and enhancing quiescence, proving that CITED2 is crucial for AML maintenance[101]. More recently, Mattes *et al*[103] have reported that loss of CITED2 impairs AML cell survival *via* p53-mediated apoptosis by interfering with the AKT signal transduction pathway. Moreover, the same authors have shown that simultaneous upregulation of CITED2 and downregulation of PU.1 enhances the lifespan of HP hematopoietic stem and progenitor cells (HPSCs), which makes them more prone to full leukemic transformation[104]. These results indicate that CITED2 plays an important role in the pathogenesis of AML and it should be considered as a target for AML therapy. Interestingly, HIF-1α was reported to play also important

roles in the self-renewal of AML leukemic stem cells and was indicated as a potential therapeutic target for eliminating these leukemic stem cells[105-107], but a possible role for CITED2 in this context was not explored.

Cited2 and lung cancer

Lung cancer is the leading cause of cancer-related death and approximately 80% of lung cancers are identified as non-small cell lung carcinoma (NSCLC)[108]. It is known that TGF-α and TGF-β are two key cytokines that regulate proliferation and quiescence, respectively, of lung epithelial cells during both normal and neoplastic lung development[109]. CITED2 plays also an important role in lung development, as demonstrated by the fact that *CITED2* knockout mice display abnormal fetal lung development[13]. Chou *et al*[110] showed that in lung cancer, CITED2 works as a molecular switch for TGF-α proliferation and TGF-β quiescence stimuli in a MYC-dependent pathway. CITED2 was shown to recruit p300 to induce MYC-p300-mediated transactivation of E2F3, leading to increased G1 to S cell cycle progression. Moreover, CITED2 was able to inhibit cellular quiescence by promoting MYC-mediated suppression of $p21^{CIP1}$[110]. Moreover, the same authors also observed that CITED2/MYC/E2F3/$p21^{CIP1}$ pathway was activated in patients with NSCLC with a poor prognosis[110].

Cited2 and cancer stem cells

The previous studies support the notion that CITED2 has context-dependent roles in cancer (Table 1). Although less extensively, CITED2 has also been shown to play a role in some other cancers, such as undifferentiated pleomorphic sarcoma, osteosarcoma, thyroid, and ovarian cancers[111-114]. CITED2 is also likely to play a role in an important aspect of tumor development related to cancer stem cells (CSCs). It is currently accepted that tumors originate from CSCs, which may derive from normal stem or progenitor cells that have lost the ability to self-regulate proliferation and quiescence[115,116]. Interestingly, an abnormal expression of core pluripotency transcription factors, such as OCT4, SOX2, NANOG, TBX3 and KLF4, has also been associated with CSCs, suggesting that the expression of pluripotent gene regulatory network factors may contribute to the conversion of normal cells into CSCs[117]. In murine embryonic stem cells, Cited2 controls the expression of Nanog, Tbx3, Klf4 and Oct4[118,119], and in adult stem cells it was associated with self-renewal, survival and quiescence[52,120,121]. Moreover, the CITED2-target gene *BMI1*, was shown to be involved in various CSC functions[122-124]. Thus, in particular circumstances, an anomalous increase of CITED2 expression may contribute to uncontrolled self-renewal and proliferation of stem cells and originate CSCs. Supporting this notion, a subset of patients with AML present an aberrantly elevated expression of CITED2 in CD34-positive leukemic cells compared to normal cells[101]. The imbalanced CITED2 expression due to a failure of PU.1 repression during normal myelopoiesis is likely to promote the initiation and maintenance of leukemia, and potentiate the establishment of a subset of multipotent leukemic stem cells[101]. On the other hand, patients with NSCLC expressing CITED2/MYC/E2F3/$p21^{CIP}$ have a poor prognosis[110], but CITED2 was demonstrated to repress the expression of CSCs markers in NSCLC-stem cells and enhance their sensitivity to ionizing radiations in combination with butyrate treatment[125]. Therefore, the potential role played by CITED2 in the generation and maintenance of CSCs may vary with the nature of the tumor. Interestingly, increasing evidence indicates that HIFs regulate the sub-populations of CSCs in BC, CRC, and AML, for instance[126]. Therefore, studies to determine whether abnormal levels of CITED2 are important in CSC functions and weather CITED2-mediated inhibition of HIF signaling is on the basis of these functions should be pursued.

CONCLUSION

Altogether, the previous studies have shown that CITED2 is expressed ubiquitously and exhibits a very high affinity for the CH1 domain of the transcriptional co-activators CBP/p300, for which it competes with HIFs. CITED2 plays an important role in inhibiting HIF-1α in some diseases reviewed here, and may play a role in many others, since altered HIF activity was reported also in stroke, heart attack, and pulmonary hypertension, for example. In the particular case of cancer, CITED2 has been reported to have both oncogenic and tumor suppressive properties, depending on the cell/tumor type and treatment, like it was also shown for HIF. A role for CITED2 in the maintenance of CSCs was also unveiled in some cancers and seems to be also context-dependent. On the other hand, HIF signaling in CSCs is well established. Despite evidence linking both CITED2 and HIF functions independently to several aspects of cancer, little data linking the interplay between these two factors

Table 1 Effects of CITED2 in different cancer

Cancer type	CITED2 effect	Biological context	Cellular effects	Interaction with HIF-1α	Ref.
Breast cancer	Overexpression	Cell lines (MCF-7; SKBR-3; MDA-MB-231; MDA-MB-468; NT2.5); primary tumor; mouse xenograft	Proliferation; migration; metastization; chemoresistance; reduced survival	Not involved	[64,65,67-69,71]
Colorectal cancer	Downregulation	Cell lines (RKO; SW480)	Migration; invasion	Not determined	[75]
Prostate cancer	Overexpression	Cell lines (LNCaP; VCaP; DU145; PC-3; C4-2B; 22RV1); primary tumor; mouse xenograft	Invasion; metastization	Not determined	[79]
Gastric cancer	Overexpression	Cell lines (MKN74; MKN28; 7901; AGS; SNU-1; SNU-5; NCI-N87; KATO-III; MNK7; MNK45); primary tumor; transgenic mice	Proliferation; cell cycle progression; clonogenicity; chemoresistance	Not determined	[82,83]
Hepatocellular carcinoma	Downregulation	Cell lines (LO2; Hep3B; HepG2; BEL7404; primary tumor	Proliferation; clonogenicity	Not determined	[87-90]
Acute myeloid leukemia	Overexpression	Primary cell cultures; cell lines (NB4; MOLM-13); transgenic mice	Cell survival; proliferation; increased quiescence of AML-LSC	Self-renewal of AML-LSC	[101,103,104]
Lung cancer	Upregulation	Cell lines (A549; H1975; CL1-0; CL1-5); primary tumor	Cell cycle progression; clonogenecity; reduced survival	Not determined	[110]

HIF: Hypoxia-inducible factor.

in these processes is available. Therefore, comprehensive studies exploring the interactions between CITED2 and HIF-1α, which are important mediators in cancer, are sorely needed. A better understanding of this interplay may potentially lead to novel strategies for the development of innovative, targeted therapies.

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MINIREVIEWS

Formulation strategies in immunotherapeutic pharmaceutical products

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Abstract

Development of immunologic-based biopharmaceutical products have strikingly increased in recent years and have made evident contributions to human health. Antibodies are the leading entity in immunotherapy, while chimeric antigen receptor T cells therapies are the advent of a novel strategy in this area. In order to enable antibody candidates or cells available as products, formulation is critical in terms of stabilize molecules or cells to achieve practical shelf life, storage and handling conditions. Here we provide a concise and contemporary review of ongoing formulation strategies and excipients used in approved antibodies and cellular therapeutic products. Excipients are categorized, and their function in formulations are discussed.

Key words: Immunotherapeutic; Pharmaceutical products; Formulation; Excipients; Cell therapy; Antibody

Core tip: In this review, we have focused on the formulation strategies and excipients that have been used in commercialized antibody products as well as the formulation concerns for immuno-cell therapy. Development of immunologic-based biopharmaceutical products have strikingly increased in recent years and have made evident contributions to human health. Antibodies are the leading entity in immunotherapy, while chimeric antigen receptor T cells therapies are the advent of a novel strategy in this area.

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INTRODUCTION

The approval of the first therapeutic monoclonal antibody (mAb) in 1986, Orthoclone OKT3, "opened the gate" of antibody therapy. Since then, more than 70 mAbs has been approved continuously and applied in both diagnose and therapeutics[1]. The performance of these products has proved to be remarkable in terms of minimized adverse effect and outstanding efficacy, which results from their unparalleled specificity and avidity. Yu *et al*[2] reported that progression-free survival and overall survival were greatly improved in lung cancer patients by immunotherapies as compared to chemotherapy without suffering the associated adverse reactions of chemo-patients. In addition, the half-life of mAbs are typically much longer than small molecules. For instance, the half-life of the anti-IgE mAB omalizumab (Xolair®) is 26 d[3]. This allows for once-monthly dosing, thereby avoiding the need of twice-daily doses of antihistamine agents for chronic idiopathic urticaria patients[3].

The year 2017 was celebrated within the pharmaceutical industry because of the approval of the first gene therapy product and the first two cellular therapy products, Yescarta and Kymriah™[4]. This historic action not only set forth the application of cellular immunotherapy but buttressed the success of biotechnology in disease treatment. Yescarta™ and Kymriah™, developed by Kite and Novartis, respectively, were based on chimeric antigen receptor (CAR) T-cell therapy of hematological cancers. In CAR T-cell therapy, patient's autologous T cells are collected and genetically modified by either viral or non-viral methods to express CARs specific for given tumor antigens. The modified cells are subsequently sorted and expanded *ex vivo* before re-infusion back into patients. CAR is a fusion of two domains: An extracellular domain for tumor antigen recognition and an intracellular signaling domain that mediates T-cell activation[5]. Recently, anti-CD19 CAR T cells have been demonstrated to be remarkably effective for the treatment of relapsed or refractory B-cell malignancies in pediatric and adult patients[5,6].

Indeed, the growing market of Ab-based drugs and the advent of CAR T cell therapy have illustrated the success of the application of basic immunology to disease treatment. However, several issues have to be addressed to improve the "drugability" of new entities and to develop more candidates into products. An approved drug product must possess stable shelf-life and to endure the stresses of handling and transportation. Thus, stability and preservability have become a major challenge to Abs and cell therapies due to their relative unstable nature. Biologics are sensitive to external conditions, such as temperature changes, agitation, moisture (for solid forms), pH changes, and exposure to interfaces or denaturants[7]. Therefore, appropriate formulation is needed to enhance the stability of active pharmaceutical ingredients to maintain their potency and safety by directly or indirectly interacting with the active pharmaceutical ingredient to prevent them from being damaged by harmful factors.

In this review, we have focused on the formulation strategies and excipients that have been used in commercialized Ab products as well as the formulation concerns for immuno-cell therapy.

FORMULATIONS AND EXCIPIENTS IN ANTIBODY-BASED BIOPHARMACEUTICAL PRODUCTS

As shown in Table 1, Ab formulations are mostly in liquid form and occasionally in solid forms such as lyophilized powders. The excipients selected for Ab formulations can be categorized into 5 classes: Sugars and polyols, amino acids, surfactants, buffer and tonicifying agents, and others (preservatives, antioxidants, and chelators) (Figure 1).

Sugars and polyols

Sugars have been identified as one of the intracellular solutes (osmolytes) that stabilize microorganisms under harsh conditions such as serious dehydration and elevated temperature. Being wisely utilized in pharmaceutical industry, sugars and polyols are effective in stabilizing therapeutic Abs thereby protecting them from aggregation, denaturation and other degradative pathways in both dried and solution states.

In solution, sugars can stabilize Abs *via* increasing their melting temperatures (Tm), raising water surface tension, excluded volume effects, and preferential hydration at high concentrations[8,9]. For instance, sorbitol has been shown to increase the Tm of human IgG and reduce its aggregation during the heating process, which is employed for viral inactivation[10]. Sek[11] studied the effect of polyols in increasing the unfolding

Table 1 List of antibody products approved by the United States Food and Drug Administration in 2018 and through May 2019. Information source: www.fda.gov and each product's package insert

Trade name	API	Yr	Sponsor	Excipients[1]	Form	Storage condition
Skyrizi	Risankizumab-rzaa	2019	Abbvie	Disodium succinate hexahydrate, polysorbate 20, sorbitol, and succinic acid	Liquid	2-8 °C, avoid light/shake/freeze
Evenity	Romosozumab-aqqg	2019	Amgen	Acetate, calcium, polysorbate 20, and sucrose	Liquid	2-8 °C, avoid light/shake/freeze
Cablivi	Caplacizumab-yhdp	2019	Ablynx/Ablynx	Citrate dihydrate, polysorbate-80, sucrose, and trisodium citrate dihydrate	Lyophilized Powder	2-8 °C, avoid light /freeze
Trogarzo	Ibalizumab-uiyk	2018	TaiMed Biologics/ Theratechnologies	L-histidine, polysorbate 80, sodium chloride, sucrose	Liquid	2-8 °C, avoid light/shake/freeze
Ilumya	Tildrakizumab	2018	Sun pharma	L-histidine, L-histidine hydrochloride monohydrate, polysorbate 80, sucrose	Liquid	2-8 °C, avoid light/shake/freeze
Crysvita	Burosumab-twza	2018	Ultragenyx pharmaceutical/kyowa hakko kirin	L-histidine, L-methionine, polysorbate 80, D-sorbitol	Liquid	2-8 °C, avoid light/shake/freeze
Aimovig	Erenumab-aooe	2018	AmgenNovartis	Acetate, polysorbate 80, sucrose	Liquid	2-8 °C, avoid light/shake/freeze
Poteligeo	Mogamulizumab-kpkc	2018	Kyowa hakko kirin	Citric acid monohydrate, glycine, polysorbate 80	Liquid	2-8 °C, avoid light/shake/freeze
Takhzyro	Lanadelumab	2018	Dyax/ Shire	Citric acid monohydrate, L-histidine, sodium chloride, sodium phosphate dibasic dihydrate	Liquid	2-8 °C, avoid light/shake/freeze
Lumoxiti	Moxetumomab pasudotox-tdfk	2018	AstraZeneca	Glycine, polysorbate 80, sodium phosphate monobasic monohydrate, sucrose	Lyophilized Powder	2-8 °C, avoid light/shake/freeze
Ajovy	Fremanezumab-vfrm	2018	Teva	Disodium ethylenediaminetetraacetic acid dihydrate (EDTA), L-histidine, L-histidine hydrochloride monohydrate, polysorbate-80, sucrose	Liquid	2-8 °C, avoid light/shake/freeze
Emgality	Galcanezumab-gnlm	2018	Eli Lilly	L-histidine, L-histidine hydrochloride monohydrate, polysorbate 80, sodium chloride	Liquid	2-8 °C, avoid light/shake/freeze
Libtayo	Cemiplimab-rwlc	2018	Regeneron/Sanofi	L-histidine, L-histidine monohydrochloride monohydrate, sucrose, L-proline, polysorbate 80	Liquid	2-8 °C, avoid light/shake/freeze

[1]Water for injection and pH adjusting reagents, such as hydrochloric acid and/or sodium/potassium hydroxide, are not specified here. API: Active pharmaceutical ingredient.

temperature of several Abs and reported that the extent of stabilization improved with increasing polyol concentration or with larger polyols conferring greater stability[11].

It has been widely demonstrated that solidifying biologics can improve the long-term storage stability of the biopharmaceutical product as well as ease shipping and storage related problems. Lyophilization or freeze-drying is the most commonly used technique to produce protein and peptide solids[12]. There are three major steps during lyophilization: Freezing, primary drying and secondary drying. During the processes, sugars and polyols can exert significant stabilizing effects *via* mechanisms such as water replacement and vitrification[13]. Moreover, sugars and polyols act as bulking agent to maintain the integrity of lyophilized "cake" structures[14].

Sucrose, trehalose, mannitol, and sorbitol are the most frequently selected additives for protein formulations, acting as the stabilizer in both solid and liquid forms as well as lyoprotectants and/or bulking agents in solid form[15]. Reducing sugars, comprised of monosaccharides and most disaccharides (including glucose, lactose, fructose, maltose, and maltodextrins) should be avoided in Ab formulations. This group of compounds can degrade Abs *via* the Maillard reaction during storage which leads to degradation and deactivation of the Abs[16,17].

Amino acids

The amino acid seems an ideal excipient in pharmaceutical development due to its natural origin, safety within the human body, and other functions that benefit formulations. Thus far, the most frequently used amino acids that stabilize Ab molecules in pharmaceutical products include histidine, arginine, and glycine. Amino acids have been reported to stabilize proteins by various mechanisms, including buffering capacity, thermal stabilization, antioxidant properties, preferential hydration and direct/indirect interaction with proteins[9,18,19].

For example, the stabilizing effect of an equimolar mixture of L-Arg and L-Glu on colloidal and conformational stability of four monoclonal antibodies (mAb1–mAb4) at different pH was examined[20]. L-Arg and L-Glu increased the aggregation temperature of all four mAbs in a concentration-dependent manner and elevated the unfolding temperature of the least thermally stable mAb3, without direct effects on the Tm1 of other mAbs. Consequently, aggregation is suppressed with increasing temperature/pH and, importantly, under accelerated stability conditions at weakly acidic to neutral pH[20].

Surfactants

Surfactants are one of the routine additives in biopharmaceutical products (Table 1). Non-ionic surfactants are formulated with Abs to specifically assist protein refolding and non-specifically suppress surface interaction-related aggregation against various stresses, including increasing temperature, freezing, dehydration, rehydration, and agitation. The fundamental pathway of the surfactant stabilization effect is to prevent surface adsorption and subsequent denaturation of Abs *via* competing with the protein for container surface, air-water interface, ice-water interface, solid-air interface and any other non-specific adsorption[9,21-23]. Certain surfactants also can directly and specifically bind noncovalently to the hydrophobic region of Abs. Stabilization results when the binding of the surfactant ligand is weaker in the non-native state than in the native state. This allows binding to hydrophobic sites of the protein to protect it from interacting with other Abs or surfaces[24]. Most commonly added surfactants are polysorbate 20, polysorbate 80, and poloxamer 188, regardless liquid or solid forms[25].

Buffer agents and tonicifying agents

Buffer systems are typically comprised of two chemical species that are related to a change in protonation state. The major function of a buffering agent system in a formulation is to provide a relatively consistent pH at which the active ingredient is physically and chemically stable. Several chemical degradation pathways are pH dependent for example, deamidation and oxidation. An arginine-acetate buffer was found to stabilize an IgG1 Ab against deamidation and aggregation at pH 4.5 to 6.0[26]. In addition, buffer agents also influence the electrostatic interaction both inter- and intra-molecularly by controlling solution pH. Otherwise, intramolecular charge repulsion can compromise the native structure of Abs, leading to protein unfolding[27]. Alternatively, intermolecular charge repulsion can protect the native structure, resulting in increasing Ab colloidal stability and solution phase stability[27,28]. Commonly used salt buffer systems are listed in Table 2.

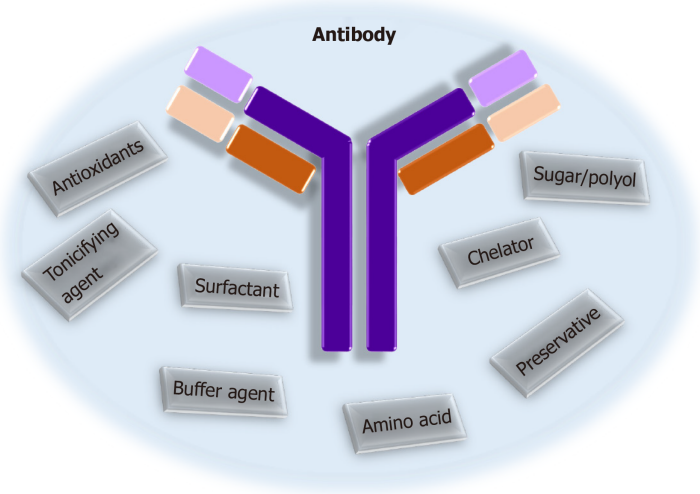

Figure 1 Cartoon of antibody formulations and excipients. Heavy chain (purple) and light chain (brown) constant regions; heavy (purple) and light (brown) antigen-binding variable regions. Excipients are depicted as gray rectangles which associate with Abs in noncovalent fashion.

Besides maintaining pH, as mentioned above, salts also can act to resolve "tonicity" (*i.e.*, osmotic pressures differences between two solutions). Sodium chloride is the most commonly used of the iso-tonicizing agents. Other than salts, excipients like mannitol, lactose, and glycerin, are also incorporated into Ab formulations (mostly parenteral) to prevent tonicity related symptoms, including pain, irritation or tissue damage at the administration site[21].

Preservatives, antioxidants, and chelators

Chelators and antioxidants are typically used to prevent the oxidation of Abs and other excipients. Several conserved amino acid residues encoded within Abs, such as methionine and cysteine, are prone to oxidative degradation. During stages of production, purification, formulation, and storage, three sources can provide oxidative molecules to product formulations, including trace metal ions from containers or handling tools (during the extended production process), hydrogen peroxide from sanitizing agents, and additional oxidant impurities from other excipients[29,30]. Besides, antimicrobials are typically added to the formulations, especially when employing multi-dose vials, as preservatives to inhibit microbial proliferation. These frequently employed antioxidants, chelators and antibiotics include edetic acid/or edetate salts (*e.g.*, EDTA), glutathione, metacresol, phenol, benzyl alcohol, benzalkonium chloride, and certain amino acids such as methionine and cysteine[25].

IMMUNO-CELL THERAPY FORMULATIONS

Currently, the typical CAR-T manufacturing process involves blood collection, apheresis, T-cell activation, gene modification, cell expansion, formulation and packaging, cryopreservation, and eventually injection into patients[31]. During these steps, cells experience multiple transportation. They also are exposed to processes such as separation, transduction, expansion and freeze-thaw. Each of the several steps of synthesis and operation require specific environments for the cells which expose them to different compositions in the formulation[32,33]. Similar to other biopharmaceutical products, cells need ancillary materials to provide necessities for stability, including non-oxidative/reducing environment, proper pH, and other critical factors[34]. However, unlike biologics, cell-based products also need nutritional components to keep them alive and to maintain robust metabolism as well as cryoprotectant agents (CPA) to protect them from the stresses caused by dramatic temperature fluctuations during their processing.

CPAs are typically necessary in cell-based products to support cells for surviving freeze-thaw processes that facilitates transportation. Often non-electrolytes are added as CPAs, including low molecular molecules such as sugars, glycerol (trehalose and sucrose) and dimethyl sulfoxide (DMSO), as well as large polymeric molecules (*e.g.*, polyvinylpyrrolidone and hydroxyethyl starch)[35]. Since the discovery of the

Table 2 Non-amino acid buffer systems frequently used in antibody parenteral products

Buffer system	Controlled pH range (25 °C)	Acid	Base	Example product
Phosphate	5.8-7.8	Monosodium phosphate	Disodium phosphate	Tysabri®
Acetate	3.8-5.8	Acetic acid	Sodium acetate	Amgevita®
Citrate	3.0-7.4	Citric acid	Sodium citrate	Humira®
Succinate	3.3-6.6	Succinic acid	Sodium succinate	Kadcyla®
Tris	7-9	Tris-HCl	Tris	Besponsa®

cryoprotective property of DMSO in 1959[36], it has been investigated and routinely employed as a cryoprotectant in cellular products. For example, DMSO is used in majority of mostly approved cell products, HPC cord blood, as well as current CAR-T cell formulations to enable short term storage and transportation between the hospital and the CAR-T cell manufacturer[37].

There are a number of potential issues that concern drug developing organizations and regulatory agencies for CAR-T cell application[38,39]. The exposure of cells to a variety of formulations during the multiple steps of processing and manufacturing may cause the final product to carry residual amounts of the unintended components. These could be potential hazards in a drug product and thus, requires risk assessment. Yet, the limited shelf life of some cellular products and the impact of extensive tests on their quality hinders the removal, or at least the assessment, of the residuals[39-41].

Another issue results from the complexity of ancillary materials or excipients. Even subtle change within culture supplies can be influential to cellular physiology and may lead to the changes in their functional characteristics and performance. Also, serum and recombinant proteins might carry pathogen contamination. Therefore, the quality and stability of ancillary/excipient materials are crucial and need to be strictly controlled[42]. Further studies to improve excipient/ancillary materials, both systemic and detailed, are urgently needed. These include determination of the correlation of excipient with cell density and process parameters (primarily freezing and thawing) as well as container-excipient compatibility. Finally, the developments of novel excipient and even new dosage forms for CAR T-cells are anticipated. For example, a recent patent reported that T cells can be kept activated *via* cross-linking when mixed with biodegradable nanospheres/microspheres[43].

CONCLUSION

The discovery and invention of immunotherapies is a milestone in the history of the battle between humans and diseases. Inactive ingredients (*e.g.*, excipients) are critical component of a successful immune-biopharmaceutical product. This review offers a brief and concise introduction to the currently used excipients and formulation strategies for antibody drugs and immune cell-based therapeutics. Knowledge about formulation compositions for Abs injectables has significantly matured, and the understanding of mechanisms of excipients is increasing. However, more dosage forms are anticipated for mAbs, especially the ones that are less or not invasive to patients, resulting in an improved patient compliance. For example, administration routes such as nasal, respiratory and oral can be promising options. As mentioned previously, the development of immune cell therapy is only in its infancy, future investigation remains. There are still many aspects of issues urgently need to be addressed by formulation scientists, such as manufacture process optimization, excipient choice, and stability of formulation or environment cells are exposed to.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Human epidermal growth factor receptor 2 positive rates in invasive lobular breast carcinoma: The Singapore experience

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Abstract

BACKGROUND

Invasive lobular carcinomas (ILC) form 5%-10% of breast cancer and rarely show overexpression of human epidermal growth factor receptor 2 (*HER2*).

AIM

To describe the prevalence and prognostic factors of *HER2* positive (*HER2*+) ILC

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in an Asian population.

METHODS
A retrospective review of patients with ILC seen between January 1985 and March 2018 at various SingHealth medical institutions was conducted. Demographic and clinical data were collected from medical records. We examined clinicopathological characteristics and survival in relation to *HER2* status.

RESULTS
A total of 864 patients were included. Prevalence of *HER2* positivity was 10.1% (87 patients). Compared with *HER2* negative (*HER2*-) ILC, *HER2*+ ILC was associated with a higher proportion of estrogen receptor negative (24.4% *vs* 5.9%, $P < 0.001$), progesterone receptor negative (*PR*-) (40.2% *vs* 24%, $P = 0.002$) and grade 3 tumours (Grade 3, 29.0% *vs* 10.2%, $P < 0.001$). Overall survival rate was poorer in patients with *HER2*+ compared to *HER2*- ILC (56.7% *vs* 72.9% alive at 10 years; hazard ratio 1.87, 95% confidence interval: 1.21-2.90, $P = 0.004$). Based on multivariate analysis, negative prognostic factors for overall survival included *HER2* positivity, *PR* negativity, older age, Indian ethnicity and higher tumour stage.

CONCLUSION
Prevalence of *HER2*+ ILC was 10.1%. *HER2*+ ILC was more likely to have poorer prognostic features such as estrogen receptor negative, *PR*- and higher tumour grade, and have a poorer survival.

Key words: Lobular breast cancer; Invasive breast cancer; Human epidermal growth factor receptor 2 positive; Singapore; Clinicopathological characteristics; Prognostic value

Core tip: We conducted a retrospective review of 864 patients with invasive lobular breast carcinoma (ILC) and examined the clinicopathological characteristics and survival in relation to human epidermal growth factor receptor 2 (*HER2*) status. Interestingly, our cohort reports a higher prevalence of *HER2* positive ILC (10.1%) as compared to some previous studies. *HER2* positive ILC was more likely to have poorer prognostic features such as estrogen receptor negative, progesterone receptor negative and higher tumour grade, and these patients have a poorer survival compared to those with *HER2* negative ILC.

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INTRODUCTION

Invasive lobular carcinomas (ILC) represent about 5%-10% of breast cancer[1-3]. Prevalence of overexpression of human epidermal growth factor receptor 2 (*HER2*) in breast cancer has been reported at 4.8%-5.1%[4,5]. The clinicopathological characteristics of *HER2* positive (*HER2*+) invasive ductal carcinomas (IDC) are known to differ from that of *HER2* negative (*HER2*-) IDC. *HER2*+ IDC is associated with estrogen receptor negativity (*ER*-), progesterone receptor negativity (*PR*-) and higher histologic grade[4,6]. A number of reports suggest that these associations are also present in ILC and that *HER2* positivity may be a prognostic factor[7-13]. However, there remains a paucity of research examining the characteristics of *HER2*+ as opposed to *HER2*- ILC, particularly in Asian populations. This study aims to investigate the prevalence and prognostic clinicopathological factors of *HER2*+ ILC.

MATERIALS AND METHODS

Study design

A retrospective review of patients with ILC seen between January 1985 and July 2018 at National Cancer Centre Singapore, Singapore General Hospital, Changi General Hospital and KK Women's and Children's Hospital was conducted. We obtained the clinical and pathological data of ILC patients from the Joint Breast Cancer Registry, our prospective database. Clinical variables included patient demographic factors such as age at diagnosis, gender, ethnicity, disease factors such as tumour side, size, grade, stage, nodal status, *ER*, *PR* and *HER2* status, as well as treatment given such as chemotherapy, radiotherapy, surgery and anti-*HER2* therapy. The study was reviewed and approved by the SingHealth Institutional Review Board CIRB Ref: 2019/2419.

Inclusion and exclusion criteria

From 1985 to 2018, 1095 patients were diagnosed with ILC. Of these, 242 patients with unknown *HER2* status were excluded from the study. Twelve patients with pathological stage 0 breast cancer were also excluded from the study. The remaining 864 patients were analysed (Figure 1).

Pathology assessment

Histopathological diagnoses of ILC were made by pathologists at various SingHealth medical institutions, namely Singapore General Hospital, Singapore; Changi General Hospital and KK Women's and Children's Hospital. Pathologic variables collected included *ER*, *PR* and *HER2* status. ASCO-CAP guidelines were used to define positivity cut-offs for the tumours as follows: A positive *ER/PR* result was defined as the presence of at least 1% of tumour cell nuclei displaying unequivocal staining of any intensity, and for *HER2*, tumour positivity was defined as > 10% of tumour cells exhibiting 3+ membrane staining. Ambiguous *HER2* cases were tested and confirmed by fluorescence *in situ* hybridization testing based on the ASCO-CAP guidelines[6-9]. In the Joint Breast Cancer Registry database, tumours were also classified into a molecular subtype as follows: Basal (*ER*-, *PR*- and *HER2*-); *HER2*+ (*ER*-, *PR*- and *HER2*+); Luminal A (*ER*- or *PR*- and *HER2*-); Luminal B (*ER*+ or *PR*+ and *HER2*+).

Statistical analysis

All demographic and clinicopathological characteristics were summarized in terms of *HER2* status, as *HER2*+ and *HER2*- ILC. Categorical and continuous variables were summarized as frequency with percentage and median [interquartile range (IQR)] respectively. Differences between *HER2*+ and *HER2*- ILC were tested using chi-squared test for categorical variables and Mann-Whitney *U* test for continuous variables.

The primary outcome overall survival (OS) was treated as time-to-event data and survival time was defined as time from date of diagnosis to date of death or date last seen. Secondary outcomes included disease-free survival (DFS) and breast cancer-specific overall survival (BCSS). DFS was treated as time-to-event data and duration of DFS was defined as duration from date of last treatment to date of relapse or date last seen or date of mortality. BCSS was treated as time-to-event data and duration of BCSS was defined as duration from date of last treatment to date last seen or date of mortality if cause of death was attributed to breast cancer. OS, DFS and BCSS were analysed for *HER2*+ and *HER2*- status using Kaplan-Meier survival analysis and were tested using log-rank test.

Univariate and multivariate Cox proportional hazard (CPH) regression analysis were used to find associations between OS and other prognostic factors in these patients with ILC. The following clinicopathological characteristics were investigated in the model: Age, ethnicity, *ER* status, *PR* status, *HER2* status, tumour size, stage, grade and treatment modalities such as chemotherapy, radiotherapy and surgery. Variables with $P < 0.03$ in the univariate CPH model were selected for multivariable model. Final multivariate CPH model was selected using stepwise, forward and backward variable selection method. Quantitative association from CPH regression model was expressed in terms hazard ratio with corresponding 95% confidence interval. Three separate CPH models were used for OS, DFS and BCSS. All statistical tests were two-sided and $P < 0.05$ was considered statistically significant. Analyses were performed using SAS Institute Inc 2013. SAS/ACCESS® 9.4 Interface to ADABAS (SAS Institute Inc., Cary, NC, United States).

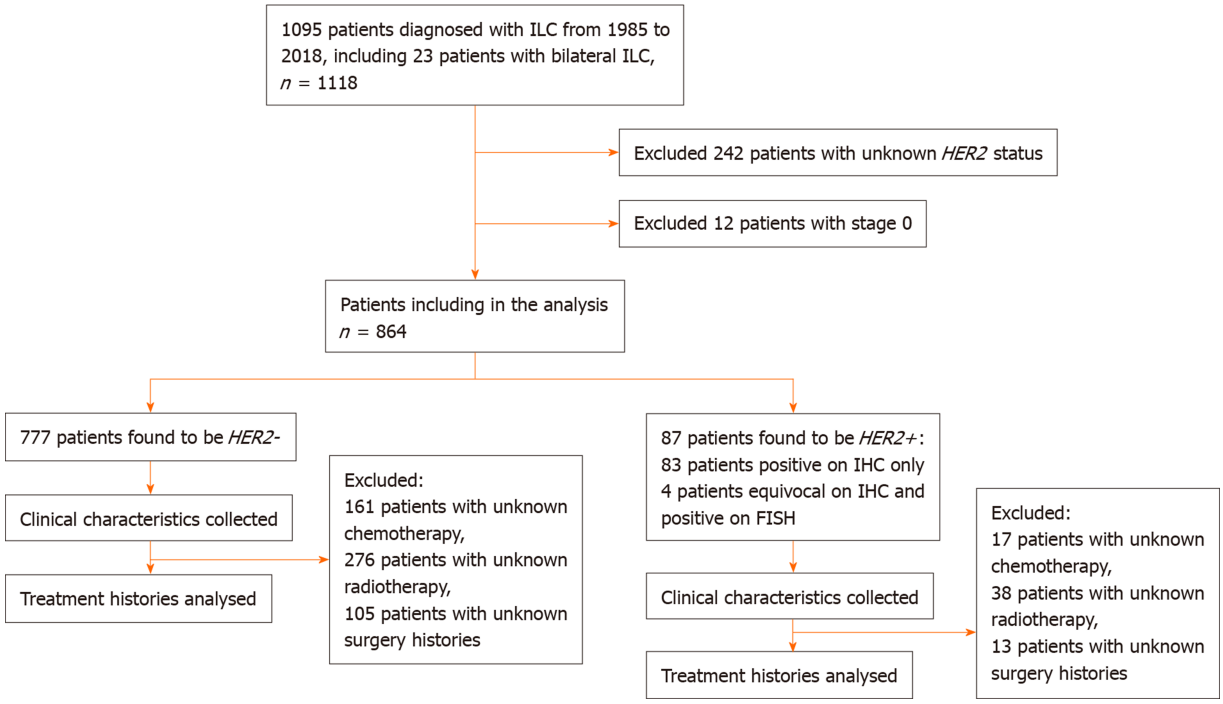

Figure 1 Consort flow diagram showing inclusion and exclusion of patients in study population. Human epidermal growth factor receptor 2 positive (*HER2*+) invasive lobular carcinomas was defined as an immunohistochemistry score of 3+ or an immunohistochemistry score of 2+ with a *HER2* to chromosome 17 ratio ≥ 2.0 for samples after 1 January 2014 and *HER2* to chromosome 17 ratio ≥ 2.2 for samples before 1 January 2014 on fluorescence *in situ* hybridization testing[4]. ILC: Invasive lobular carcinomas; *HER2*: Human epidermal growth factor receptor 2; IHC: Immunohistochemistry; FISH: Fluorescence *in situ* hybridization; *HER2*+: Human epidermal growth factor receptor 2 positive; *HER2*-: Human epidermal growth factor receptor 2 negative.

RESULTS

Clinical characteristics

A total of 864 patients with ILC were included in the analysis. Study population characteristics are shown in Table 1. Of note, a total of 87 (10.1%) were diagnosed with *HER2*+ ILC. Compared with *HER2*- ILC, *HER2*+ ILC was associated with a higher proportion of *ER*- (24.4% *vs* 5.9%, $P < 0.001$), *PR*- negative (40.2% *vs* 24%, $P = 0.002$) and grade 3 tumours (Grade 3, 29.0% *vs* 10.2%, $P < 0.001$) (Table 1).

Treatment characteristics

Among the 87 patients with *HER*2+ ILC, 47 (54.0%) received *HER*2-directed therapy, 12 (13.8%) did not receive *HER*2-directed therapy and treatment data was not available for the remaining 28 (32.2%) patients. Of the patients who did not receive *HER*2-directed therapy, reasons cited upon review of clinical charts included cardiac comorbidities, poor performance status, very early stage cancer, refusal of therapy or lack of access to therapy in the years prior to the availability of *HER*2-directed therapy.

Survival outcomes

The median survival time was 2.95 (IQR: 1.89-8.87) years and 4.16 (IQR: 1.84-8.32) years respectively for *HER2*+ and *HER2*– ILC patients ($P = 0.315$). The 5-year and 10-year OS rates were 68.3% (59/87 patients) and 56.7% (49/87 patients) respectively in *HER2*+ patients and 83.4% (648/777 patients) and 72.9% (566/777 patients) respectively in *HER2*- patients (log-rank $P = 0.004$). The 5-year and 10-year BCSS and DFS rates in *HER2*+ and *HER2*- ILC patients are also shown in Figure 2.

We performed a univariate and multivariate CPH regression analysis of OS in all 864 ILC patients. Based on the multivariate analysis, significant negative prognostic factors were *HER2*+, age, ethnicity and stage. *HER2*+ and luminal B molecular subtypes also had also notably poorer OS compared to Luminal A subtype (Table 2, Figure 3). Additional univariate and multivariate CPH regression analyses of BCSS and DFS demonstrated that *HER2* positivity remained a significant negative prognostic factor for BCSS and DFS on both the univariate and multivariate analysis (Tables 3 and 4).

Table 1 Clinical and histopathological characteristics of human epidermal growth factor receptor 2 positive and human epidermal growth factor receptor 2 negative invasive lobular carcinomas patients, *n* (%)

Characteristics	*HER2*+ (*n* = 87)	*HER2*- (*n* = 777)	Total (*n* = 864)	*P* value
Age (yr)				1.000
≤ 50	30 (34.5)	272 (35.0)	302 (35.0)	
> 50	57 (65.5)	505 (65.0)	562 (65.0)	
Ethnicity				0.594
Chinese	68 (78.2)	558 (72.1)	626 (72.7)	
Indian	4 (4.6)	60 (7.8)	64 (7.4)	
Malay	8 (9.2)	68 (8.8)	76 (8.8)	
Others	7 (8.0)	88 (11.4)	95 (11.0)	
ER				< 0.001
Negative	21 (24.4)	46 (5.9)	67 (7.8)	
Positive	65 (75.6)	730 (94.1)	795 (7.8)	
PR				0.002
Negative	35 (40.2)	185 (24.0)	220 (25.6)	
Positive	52 (59.8)	587 (76.0)	639 (74.4)	
Tumour size				0.765
0.1-2 cm	21 (41.2)	230 (38.7)	251 (38.9)	
> 2 cm	30 (58.8)	365 (61.3)	395 (61.1)	
Tumour grade				<0.001
Grade 1	7 (10.1)	148 (22.5)	155 (21.3)	
Grade 2	42 (60.9)	443 (67.3)	485 (66.7)	
Grade 3	20 (29.0)	67 (10.2)	87 (12.0)	
Tumour stage				0.066
Stage 1	20 (24.1)	216 (30.3)	236 (29.7)	
Stage 2	25 (30.1)	267 (37.5)	292 (36.7)	
Stage 3	27 (32.5)	179 (25.1)	206 (25.9)	
Stage 4	11 (13.3)	50 (7.0)	61 (7.7)	
Treatment				
Chemotherapy[1]	50 (66.7)	390 (54.2)	440 (55.3)	0.038
With *HER2* therapy	47 (54.0)	- -	47 (54.0)	
No *HER2* therapy	12 (13.8)	- -	12 (13.8)	
Unknown if any *HER2* therapy	28 (32.2)	- -	28 (32.2)	
Radiotherapy[2]	47 (62.7)	404 (56.1)	451 (56.7)	0.276
Surgery[3]	73 (92.4)	690 (92.1)	763 (92.1)	0.929

[1]There were 69 patients with unknown chemotherapy histories which were excluded from analysis.
[2]There were 69 patients with unknown radiotherapy histories which were excluded from analysis.
[3]There were 36 patients with unknown surgery histories which were excluded from analysis. *HER2*: Human epidermal growth factor receptor 2; *HER2*+: Human epidermal growth factor receptor 2 positive; *HER2*-: Human epidermal growth factor receptor 2 negative; *ER*: Estrogen receptor; *PR*: Progesterone receptor.

DISCUSSION

Interestingly, although most ILC patients have *HER2*- tumours, our cohort reports a higher prevalence of *HER2*+ ILC (10.1%) as compared to some previous studies[1-5]. The largest known study to date of 85048 ILC patients in the United States SEERS database found a *HER2*+ prevalence of only 4.8%[5]. Given that our study is one of the first few to describe prevalence of *HER2*+ ILC in Asian populations, this may suggest differences across ethnic and geographical populations, although further studies are required to validate this finding.

In our cohort, *HER2*+ ILC was significantly associated with *ER* negativity, *PR* negativity and higher tumour grade. This affirms findings in a previous study which concluded that *HER2* positivity had an inverse relationship with *ER* and *PR* expression in ILC[10]. In the same study, *PR* negativity was notably more common than *ER* negativity in *HER2*+ ILC. This was also seen in our study with the frequency of

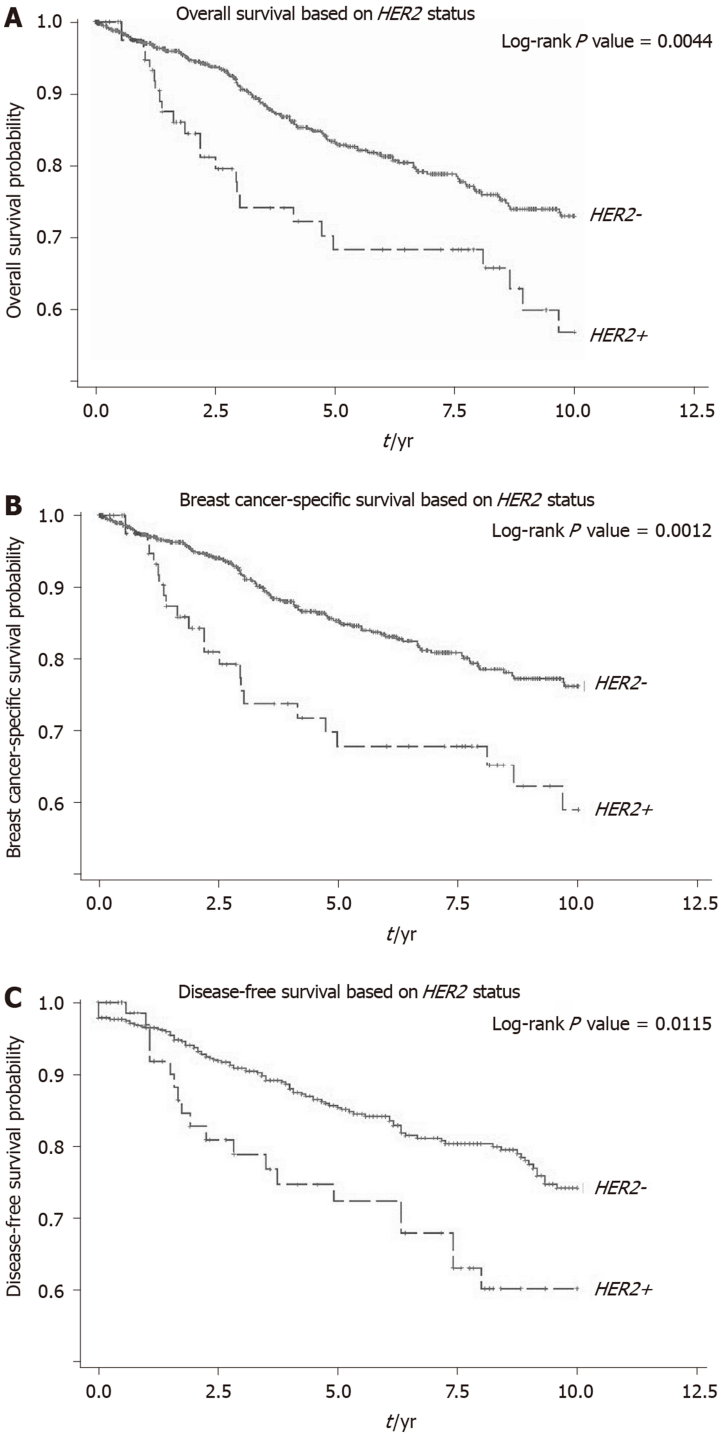

Figure 2 Kaplan–Meier estimates of difference in 5-yr and 10-yr overall survival, breast cancer-specific survival and disease-free survival in all 864 human epidermal growth factor receptor 2 positive and human epidermal growth factor receptor 2 negative invasive lobular carcinomas patients by human epidermal growth factor receptor 2 status. A: Overall survival; B: Breast cancer-specific survival; C: Disease-free survival for human epidermal growth factor receptor 2 positive and human epidermal growth factor receptor 2 negative invasive lobular carcinomas patients. *HER2*: Human epidermal growth factor receptor 2; *HER2*+: Human epidermal growth factor receptor 2 positive; *HER2*-: Human epidermal growth factor receptor 2 negative.

PR- being nearly twice that of *ER*- in the *HER2*+ population. Our study reports a higher tumour grade in *HER2*+ ILC patients. This is not consistent with findings from previous studies which did not find significant associations with *HER2* positivity and tumour grade or size[11-14]. We hypothesize that this may be due the smaller sample sizes in those studies and the heterogeneity of *HER2*+ ILC[15,16].

Our study also demonstrates poorer survival rates in *HER2*+ ILC as compared to *HER2*- ILC for OS, BCSS and DFS. On exploratory analyses of molecular subtypes, both *HER2*+ and luminal B molecular subtypes reflected this poorer OS, corroborating

Table 2 Univariate and multivariate Cox proportional hazard regression analysis for overall survival among all 864 invasive lobular carcinomas patients

Characteristics	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age (reference: ≤ 50 yr)						
> 50 yr	2.32	1.68-3.20	< 0.001	2.17	1.37-3.44	< 0.001
Ethnicity (reference: Chinese)			< 0.001[1]			0.001[1]
Indian	2.53	1.62-3.94	< 0.001	3.41	1.78-6.54	< 0.001
Malay	0.95	0.50-1.82	0.889	0.98	0.42-2.29	0.961
Others	0.40	0.15-1.08	0.070	0.64	0.19-2.12	0.462
ER (reference: Negative)						
Positive	0.74	0.44-1.24	0.255			
PR (reference: Negative)						
Positive	0.62	0.44-0.87	0.005	0.57	0.35-0.91	0.018
HER2 (reference: Negative)						
Positive	1.87	1.21-2.90	0.005	2.14	1.16-3.95	0.016
Tumour size (reference: ≤ 2 cm)						
> 2 cm	2.43	1.45-4.06	< 0.001			
Tumour stage (reference: Stage 1)			< 0.001[1]			< 0.001[1]
Stage 2	2.33	1.09-4.99	0.030	1.75	0.76-4.03	0.191
Stage 3	6.98	3.42-14.25	< 0.001	4.52	2.06-9.89	< 0.001
Stage 4	61.82	29.73-128.57	< 0.001	41.74	17.95-97.04	< 0.001
Tumor grade (reference: Grade 1)			< 0.001[1]			0.075[1]
Grade 2	1.45	0.83-1.89	0.190	1.05	0.57-1.93	0.877
Grade 3	4.72	2.55-8.74	< 0.001	1.89	0.93-3.84	0.079
Chemotherapy (reference: No)						
Yes	0.97	0.69-1.37	0.866			
Surgery (reference: No)						
Yes	0.06	0.04-0.09	< 0.001			
Radiotherapy (reference: No)						
Yes	0.89	0.63-1.27	0.518			
Molecular subtype (reference: Luminal A)			0.025[1]			0.002[1]
Basal	1.52	0.79-2.90	0.206	1.13	0.38-3.29	0.830
HER2 positive	2.08	0.85-5.10	0.108	4.21	1.43-12.44	0.009
Luminal B	1.89	1.16-3.07	0.011	2.52	1.41-4.49	0.002

[1]Refers to type 3 *P* value. HR: Hazard ratio; CI: Confidence interval; *HER2*: Human epidermal growth factor receptor 2; *ER*: Estrogen receptor; *PR*: Progesterone receptor.

with a separate study which showed similar survival outcomes for the different molecular subtypes of ILC[17]. One possible biological explanation for poorer survival rates in *HER2*+ ILC is a synergistic effect of *HER2* and cadherin 1 mutations which promotes tumourigenesis and early relapses in *HER2*+ ILC[18]. The finding of Indian ethnicity being a poorer prognostic factor for ILC on multivariate analysis also deserves further validation in a larger sample size as they formed < 5% of patients in this cohort, making it challenging to draw definitive conclusions.

Due to the retrospective nature of this study, missing data limited our ability to perform analyses on treatments received with regards to survival outcomes. Prospective studies with larger long-term follow-up sample sizes are needed to validate our observations in this study.

In conclusion, our study demonstrates the prevalence of *HER2*+ ILC to be 10.1%. *HER2*+ ILC patients were more likely to have poorer prognostic features such as *ER*-, *PR*- and higher tumour grade. Lastly, patients with *HER2*+ ILC had poorer OS, BCSS and DFS compared to those with *HER2*- ILC. These findings warrant further prospective studies to validate observation and investigate the benefit of various treatment modalities to improve outcomes in *HER2*+ ILC.

Table 3 Univariate and multivariate Cox proportional hazard regression analysis for breast cancer-specific survival among all 864 invasive lobular carcinomas patients

Characteristics	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age (reference: ≤ 50 yr)						
> 50 yr	2.16	1.53-3.05	< 0.001			
Ethnicity (reference: Chinese)			< 0.001[1]			0.004[1]
Indian	2.60	1.63-4.14	< 0.001	2.55	1.28-5.05	0.008
Malay	0.89	0.43-1.82	0.744	1.07	0.43-2.67	0.885
Others	0.32	0.10-1.02	0.054	0.19	0.04-0.84	0.028
ER (reference: Negative)						
Positive	0.72	0.42-1.26	0.255			
PR (reference: Negative)						
Positive	0.61	0.42-0.88	0.008	0.40	0.23-0.70	0.001
HER2 (reference: Negative)						
Positive	2.08	1.32-3.26	0.002			
Molecular subtype (reference: Luminal A)			0.011[1]			0.004[1]
Basal	1.49	0.72-3.07	0.281	1.16	0.36-3.77	0.801
HER2+	2.34	0.95-5.74	0.064	3.74	1.26-11.09	0.018
Luminal B	2.08	1.26-3.44	0.004	2.79	1.44-5.37	0.002
Tumour size (reference: ≤ 2 cm)						
> 2 cm	2.76	1.53-4.97	< 0.001			
Tumour stage (reference: Stage 1)			< 0.001[1]			< 0.001
Stage 2	3.11	1.09-8.92	0.034	2.19	0.74-6.49	0.159
Stage 3	13.02	4.89-34.68	< 0.001	6.49	2.35-17.89	< 0.001
Stage 4	117.79	43.5-317.87	< 0.001	56.27	18.44-171.68	< 0.001
Tumor grade (reference: Grade 1)			< 0.001[1]			0.001[1]
Grade 2	1.89	0.96-3.75	0.066	1.63	0.78-3.44	0.196
Grade 3	7.10	3.44-14.64	< 0.001	4.16	1.80-9.62	0.001
Chemotherapy (reference: No)						
Yes	1.23	0.84-1.80	0.290			
Surgery (reference: No)						
Yes	0.06	0.04-0.08	< 0.001	0.23	0.11-0.51	< 0.001
Radiotherapy (reference: No)						
Yes	0.94	0.65-1.37	0.758			

[1]Refers to type 3 *P* value. HR: Hazard ratio; CI: Confidence interval; *HER2*: Human epidermal growth factor receptor 2; *ER*: Estrogen receptor; *PR*: Progesterone receptor; *HER2*+: Human epidermal growth factor receptor 2 positive.

Table 4 Univariate and multivariate Cox proportional hazard regression analysis for disease-free survival among all 864 invasive lobular carcinomas patients

Characteristics	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age (reference: ≤ 50 yr)						
> 50 yr	1.60	1.11-2.30	0.012	1.63	1.04-2.55	0.033
Ethnicity (reference: Chinese)			0.001			
Indian	2.61	1.52-4.48	< 0.001			
Malay	0.99	0.48-2.05	0.984			
Others	1.99	1.10-3.58	0.022			
ER (reference: Negative)						
Positive	1.04	0.57-1.90	0.886			
PR (reference: Negative)						
Positive	0.97	0.65-1.43	0.876			
HER2 (reference: Negative)						
Positive	1.68	1.04-2.71	0.03			
Molecular subtype (reference: Luminal A)			0.217[1]			
Basal	0.98	0.45-2.12	0.965			
HER2+	1.69	0.62-4.61	0.304			
Luminal B	1.67	0.98-2.83	0.058			
Tumour size (reference: ≤ 2 cm)						
> 2 cm	2.02	1.26-3.25	0.004			
Tumour stage (reference: Stage 1)			< 0.001[1]			< 0.001[1]
Stage 2	1.92	1.05-3.53	0.035	1.66	0.83-3.28	0.149
Stage 3	5.66	3.21-9.98	< 0.001	5.26	2.76-10.03	< 0.001
Stage 4	0.62	0.04-10.84	0.745	0.71	0.04-12.61	0.813
Tumor grade (reference: Grade 1)			< 0.001[1]			0.013[1]
Grade 2	1.79	1.02-3.16	0.044	1.32	0.73-2.40	0.357
Grade 3	3.72	1.89-7.34	< 0.001	2.69	1.32-5.50	0.007
Chemotherapy (reference: No)						
Yes	1.64	1.12-2.42	0.011			
Surgery (reference: No)						
Yes	0.14	0.08-0.23	< 0.001			
Radiotherapy (reference: No)						
Yes	1.57	1.05-2.34	0.028			

[1]Refers to type 3 *P* value. HR: Hazard ratio; CI: Confidence interval; *HER2*: Human epidermal growth factor receptor 2; *ER*: Estrogen receptor; *PR*: Progesterone receptor; *HER2*+: Human epidermal growth factor receptor 2 positive.

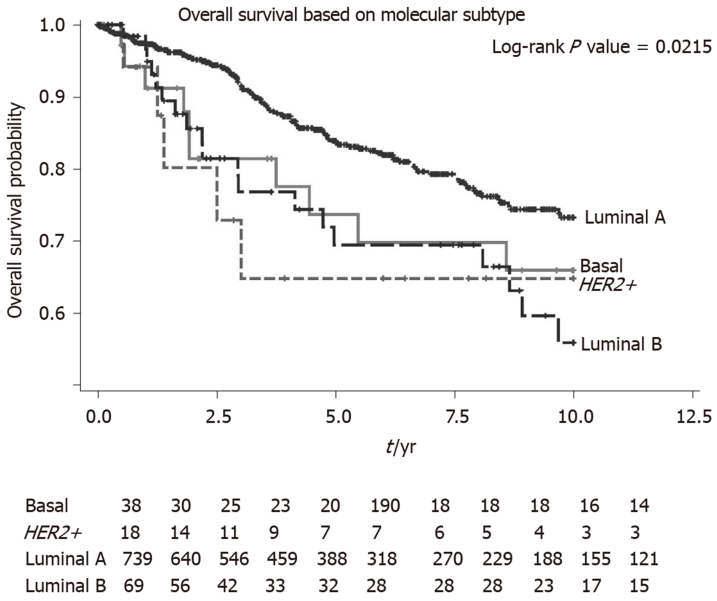

Figure 3 Overall survival of all Invasive lobular carcinomas patients by molecular subtype. Basal: Estrogen receptor (*ER*), progesterone receptor (*PR*) and human epidermal growth factor receptor 2 (*HER2*) negative; *HER2*+: *ER*, *PR* negative and *HER2* positive; Luminal A: *ER* or *PR* positive and *HER2* negative; Luminal B: *ER* or *PR* positive and *HER2* positive. *HER2*+: Human epidermal growth factor receptor 2 positive.

ARTICLE HIGHLIGHTS

Research background

Invasive lobular carcinomas (ILC) represent about 5%-10% of breast cancer. Prevalence of overexpression of human epidermal growth factor receptor 2 (*HER2*) in breast cancer has been reported at 4.8%-5.1%. The clinicopathological characteristics of *HER2* positive (*HER2*+) invasive ductal carcinomas are known to differ from that of *HER2* negative (*HER2*-) invasive ductal carcinomas. However, there remains a paucity of research examining the characteristics of *HER2*+ as opposed to *HER2*- ILC, particularly in Asian populations.

Research motivation

This study compares the clinicopathological characteristics of *HER2*+ and *HER2*- ILC to assess the differences in survival probability between the two groups.

Research objectives

This study aims to investigate the prevalence and prognostic clinicopathological factors of *HER2*+ ILC in an Asian population.

Research methods

A retrospective review of patients with ILC seen between January 1985 and March 2018 at various SingHealth medical institutions was conducted. Demographic and clinical data were collected from medical records. We examined clinicopathological characteristics and survival in relation to *HER2* status. Differences between *HER2*+ and *HER2*- ILC were tested using chi-squared test for categorical variables and Mann-Whitney *U* test for continuous variables. Overall survival (OS), disease-free survival (DFS) and breast cancer-specific overall survival (BCSS) were analyzed for *HER2*+ and *HER2*- status using Kaplan-Meier survival analysis and were tested using log-rank test. All statistical tests were two-sided and $P < 0.05$ was considered statistically significant.

Research results

Interestingly, although most ILC patients have *HER2*- tumours, our cohort reports a higher prevalence of *HER2*+ ILC (10.1%) as compared to some previous studies. The median survival time was 2.95 (interquartile range: 1.89-8.87) years and 4.16 (interquartile range: 1.84-8.32) years respectively for *HER2*+ and *HER2*- ILC patients ($P = 0.315$). Based on the multivariate analysis, significant negative prognostic factors were *HER2*+, age, ethnicity and Stage. *HER2*+ and Luminal B molecular subtypes also had also notably poorer OS compared to Luminal A subtype. Additional univariate and multivariate Cox proportional hazard regression analyses of BCSS and DFS demonstrated that *HER2* positivity remained a significant negative prognostic factor for BCSS and DFS on both the univariate and multivariate analysis.

Research conclusions

In conclusion, our study demonstrates the prevalence of *HER2*+ ILC to be 10.1%. *HER2*+ ILC patients were more likely to have poorer prognostic features such as estrogen receptor

negativity, progesterone receptor negativity and higher tumour grade. Lastly, patients with *HER2*+ ILC had poorer OS, BCSS and DFS compared to those with *HER2*- ILC.

Research perspectives

The findings from our study warrant further prospective studies to validate observation and investigate the benefit of various treatment modalities to improve outcomes in *HER2*+ ILC.

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SYSTEMATIC REVIEWS

Impact of primary tumour location on colorectal liver metastases: A systematic review

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Abstract

BACKGROUND

Colorectal cancer (CRC) is the third most common cause of cancer-related death worldwide. Despite significant advances in screening, surgical management and adjuvant therapies, average 5-year survival seldom exceeds 60% in most developed nations. Metastatic disease represents the primary cause of mortality in patients with CRC, and the liver is the most common location for distant tumour spread. Up to 25% of patients are found to have synchronous liver metastases at the time of diagnosis and a further 30%-40% will develop metachronous disease in the course of follow-up. It has been suggested that primary tumour location [right side versus left side, primary tumour location (PTL)] can influence oncological outcomes in this patient group and that this should be considered in prognostic models and therapeutic decision-making algorithms. This suggestion is not universally accepted and there have been conflicting reports in the literature to date.

AIM

To provide a comprehensive summary of the available evidence regarding the impact of PTL on oncological outcomes in patients with colorectal cancer liver metastases (CRCLM).

METHODS

MEDLINE, EMBASE and COCHRANE were searched for relevant publications using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses methodology. Data on oncological outcomes was then extracted from full text articles that met the predefined inclusion criteria.

RESULTS

A total of 41 studies were identified that met predefined inclusion criteria for this review. In 21 out of 38 studies that provided data on overall survival, a statistically significant improvement in overall survival was reported in patients

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with left sided primary tumours. These studies included a total of 13897 patients compared with 4306 patients in the studies that did not show a significant difference. Eight studies noted a similar trend towards improved disease-free or progression-free survival. Several authors observed distinct patterns of relapse after treatment of hepatic metastases according to PTL; for example hepatic recurrence after treatment of CRCLM appears to occur more aggressively with right-sided CRC.

CONCLUSION
Taken together, the findings of the present review indicate that PTL may have a role as an independent prognostic factor when determining treatment and disease surveillance strategies in CRC. The mechanisms responsible for this variation remain poorly understood, but are likely to relate to molecular, histological and embryological differences, as well as inherent differences in therapeutic sensitivity.

Key words: Liver metastasis; Colorectal cancer; Location; Primary tumour; Outcome

Core tip: Primary tumour location is associated with differing oncological outcomes and patterns of hepatic metastatic behaviour in patients with colorectal cancer liver metastases. Specifically, this systematic review indicates that there is improved overall survival in patients undergoing treatment for colorectal cancer liver metastases with left-sided colorectal cancer (CRC), compared with right-sided CRC. These findings suggest that primary tumour location may have a role in developing more individually-tailored staging, treatment and surveillance strategies for patients with CRC in the future. Current chemotherapeutic regimens may require additional modification(s) to take into account the fundamental molecular and embryological differences that underpin primary tumour sidedness.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer subtype world-wide with over 1 million new cases diagnosed in 2018[1]. Metastatic disease represents the primary cause of mortality in CRC, and up to 25% of patients are found to have synchronous metastases at the time of diagnosis. A further 40% will develop metachronous disease and approximately 25%-30% of patients will develop liver metastases in the course of follow-up[2]. Indications for curative intent treatment of CRC liver metastases (CRCLM) have expanded rapidly over the last three decades, and several key factors have led to improvements in outcome, notably enhanced radiological detection, improved chemotherapeutic efficacy and more aggressive surgical treatment[3]. With modern combined-modality treatment approaches, 5-year survival in excess of 50% has been reported in selected patients with CRCLM[4]. Clinico-pathological factors believed to be associated with worse oncological outcome in CRCLM include the presence of synchronous metastases, bi-lobar liver involvement, metastases > 5 cm in size, and the presence of extra-hepatic disease[4,5]. It has also been suggested by a number of authors that primary tumour location (PTL) - right side versus left side, can influence patterns of hepatic metastatic dissemination and survival[6,7]. For example, a number of studies have demonstrated inferior oncological outcome in patients undergoing surgical resection of CRCLM with right-sided versus left-sided colonic primary tumours[8-10]. This has not been a consistent observation, and others have shown no clear association[11,12]. The aim of the present systematic review is to provide a summary of the available evidence on the impact of PTL on oncological outcomes in patients with CRCLM.

MATERIALS AND METHODS

Identification of studies

An electronic literature search was carried out using MEDLINE (1965 to March 2020), EMBASE (1980 to March 2020) and the Cochrane Library databases. The medical subject heading terms and key words used are as follows: "Colon" or "rectal cancer", "liver metastasis" or "liver metastases" or "hepatic metastasis" or "hepatic metastases" and "left" and "Right". Studies, abstracts and citations were scanned for relevance. The latest date of this search was 27 March 2020. The publications deemed relevant were read in full and assessed for inclusion and their references scanned to identify papers not identified in the initial search.

Inclusion criteria

The methodology was designed around the "Preferred Reporting Items for Systematic Reviews and Meta-Analyses" recommendations for improving the standard of systematic reviews[13].

Studies meeting the following criteria were included for review: (1) Language: Full article accessible in English language only; Conference abstracts only were excluded. (2) Patient population: Studies reporting outcomes in ≥ 10 male/female patients aged ≥ 18 years with colorectal cancer and liver metastases. Where multiple publications were identified covering overlapping periods of time from the same institution/research group, the most recent and/or relevant data were selected for inclusion, and (3) Outcome measures: Studies were included if they reported oncological outcome data such as overall survival (OS), progression-free survival (PFS), recurrence-free survival (RFS) or disease-free survival (DFS). Studies reporting oncological outcomes for metastatic colorectal cancer were excluded if results were not reported for liver metastases specifically. Patients with metastases at multiple sites were included if one site was liver.

Data extraction

Three authors (Bingham G, Shetye A, Suresh R) independently extracted the following data from eligible studies: First author, year of publication, country of origin, study type, number of patients by gender, site of primary and age, primary study endpoint(s), secondary endpoint(s), extent and distribution of liver metastases, follow-up duration, adjuvant/neoadjuvant management, overall survival, progression-free survival, recurrence-free survival. Where there was uncertainty regarding inclusion a second author was consulted for consensus. All papers included were graded according to level of evidence using the system proposed by the Scottish Intercollegiate Guidelines Network[14]. A Preferred Reporting Items for Systematic Reviews and Meta-Analyses flow-diagram summarising the above search strategy is provided in Figure 1.

RESULTS

A total of 4094 potentially relevant publications were initially identified through the search strategy summarised in Figure 1. After screening of titles and abstracts, 3700 publications were withdrawn, leaving 394 articles for full text review. A reference search from these articles identified a further 26 studies of potential relevance. Of the 420 full text publications that were evaluated, 41 studies, including a total of 18426 patients, were found to meet our predefined inclusion criteria and were included in the review process. Study characteristics from these 41 studies are summarised in Table 1. Study population in these studies ranged from 24 to 3125 patients. Studies comprised two cohort prospective studies[15,16] (evidence level 2+) and 39 retrospective studies (evidence level 2+ - 2++)[8-12,17-50], this included 6 papers with pooled analysis (evidence level 2+-2++)[10,12,15,16,18,49]. There were no randomised controlled trials.

Overall survival

Data on the influence of PTL on OS in CRCLM was provided by 38 of the studies included for review, including a total of 18203 patients. In 21 of these studies (13897 patients -76.3% of the total patient population captured) a statistically significant trend was observed with improved OS in patients with left sided primary tumours undergoing treatment for CRCLM (*l*-CRCLM). For example, Wang *et al*[17] in their study of 1508 patients receiving surgical treatment for synchronous CRCLM, of which 593 had right sided primary colorectal tumours (*r*-CRCLM), found a significant difference in 5-year OS between left and right sided primaries (*l*-CRCLM 40.1%, *r*-CRCLM 24.6%, $P < 0.001$). They also found that patients with *r*-CRCLM were more

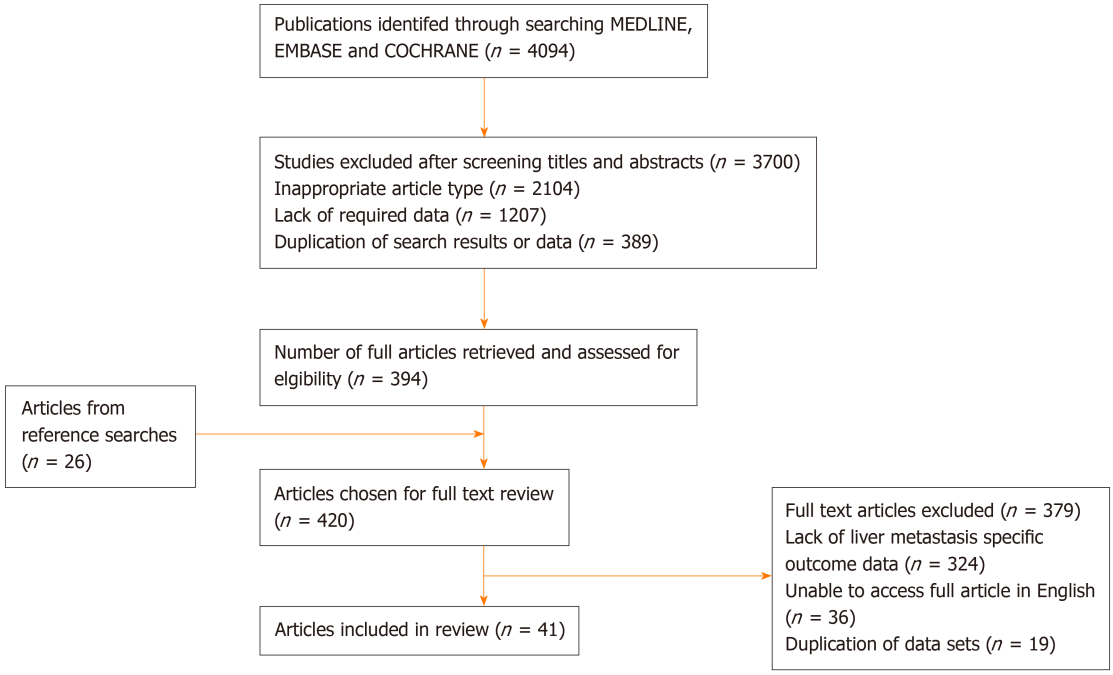

Figure 1 Preferred Reporting Items for Systematic Reviews and Meta-Analyses diagram summarising study selection process.

likely to be T4 (31.3% *vs* 20.1%, $P < 0.001$) N2 (42.5% *vs* 31.8%, $P < 0.001$), and poorly differentiated (30.5% *vs* 15.1%, $P < 0.001$). Creasy *et al*[9] in a similar cohort of 907 patients (36% with right sided primaries) undergoing hepatic resection found a found a median OS of 5.2 years in *l*-CRCLM compared with 3.6 years with in *r*-CRCLM ($P = 0.004$), with a hazard ratio (HR) of 1.22 ($P = 0.028$) on multivariate analysis. In their population database study of 3125 patients in Sweden Norén *et al*[18] found that *l*-CRCLM extended median OS by 4 mo ($P = 0.02$) compared with *r*-CRCLM. In addition, the authors reported enhanced 5-year OS (45.8% *vs* 44.5% $P = 0.02$), with a HR of 0.75 for *l*-CRCLM ($P < 0.001$).

A further 17 studies with 4306 patients found no statistically significant difference in OS between the two groups, but there was a trend towards longer OS in patients with *l*-CRCLM on the whole. For example, Gasser *et al*[12] found patients with a *l*-CRCLM had 22 mo longer median overall survival compared to *r*-CRCLM ($P = 0.051$). This contrasts with only 2 studies that showed lower OS in patients with *l*-CRCLM study, Dulundu *et al*[19] and Viganò *et al*[20], but neither with statistical significance ($P = 0.072$ and $P < 0.05$ respectively). These results are summarised in Table 2.

Disease-free survival

Benefit in DFS was also suggested, but not as convincingly as OS. This data was more sparsely provided, as some authors opted to alternatively provide PFS. Four studies including 3013 patients showed improved DFS in *l*-CRCLM. Russolillo *et al*[21] found improved median DFS by almost 1 year (32.7 mo *vs* 20.8 mo, $P = 0.002$) in their 364 patients with *l*-CRCLM (*vs* 322 patients with *r*-CRCLM) when assessing patterns of recurrence and survival following resection of liver metastases. In 2017 Heise *et al*[22] reported a DFS benefit in patients with *l*-CRCLM undergoing repeat hepatectomy after recurrence of colorectal cancer (HR: 0.19, $P = 0.001$). Liao *et al*[10] studied 1442 patients with stage III CRC who went on to develop CRCLM, and found that patients with left-sided colon cancer had better 3-year DFS (70.9% *vs* 66.5%, $P = 0.033$) compared to those with *r*-CRCLM.

In contrast to these observations, only one study by Sasaki *et al*[23] found significantly improved 3-year DFS in patients with *r*-CRCLM (28% *vs* 20.2%, $P = 0.001$) in their study of 426 patients who were undergoing curative intent hepatectomy.

Thirteen studies with 3423 patients showed no significant difference in DFS between *l*-CRCLM and *r*-CRCLM. These results are summarised in Table 3.

Progression-free survival

Only five publications provided data on PFS, and these data are summarised in Table 4. These studies including 2805 patients showed significantly improved PFS in *l*-CRCLM versus *r*-CRCLM. For example, de Haas *et al*[24] showed in 726 patients

Table 1 Summary of characteristics of included studies

Ref.	Year	Country of study	Number of patients included	Median follow up (mo)	Primary tumour location (*n*)			Median age (yr)		
					L	R	Rectum	L	R	Rectum
Zhou *et al*[27]	2017	China	295	24	89	94	112	57	59	61
Zhang *et al*[30]	2017	China	194	12	144	50		60	55.5	
Makowiec *et al*[37]	2018	Germany	221		158	63		64	65	
Chafai *et al*[29]	2005	Australia	398	26.8	277	71				
Rougier *et al*[15]	1995	France	537		197	117	223			
Wang *et al*[38]	2018	China	420	26	334	86		57	58.5	
Gu *et al*[28]	2018	China	102		51	51		63	61.5	
Gasser *et al*[12]	2019	Austria	259	38.1	200	59		64	66	
de Haas *et al*[24]	2010	France	750	39	413	154	200			
Marques *et al*[11]	2018	Brazil	151	42	124	27		57	61	
Russolillo *et al*[21]	2019	Italy	686	81	364	322		63	66	
Umeda *et al*[34]	2013	Japan	100	36	40	23	37			
Zheng *et al*[31]	2018	China	318		233	85				
Mavros *et al*[39]	2013	United States	97	26.4	44	24	27			
Viganò *et al*[20]	2014	Italy	749	51.4	63	87	48			
Connor *et al*[40]	2016	Canada	63	31.5	27	10	21			
Eefsen *et al*[41]	2015	Denmark	254	44.6	125	51	78			
Schirripa *et al*[33]	2015	Italy	309	45.6	138	87	82			
Loosen *et al*[42]	2018	Germany	125		102	23				
Amikura *et al*[32]	2018	Japan	342	52.7	236	106				
Yamashita *et al*[26]	2018	United States	725	27/41	487	238		56	58	
Dulundu *et al*[19]	2017	Turkey	108		40	24	44	58.5	63.2	63.8
Creasy *et al*[9]	2016	United States	907	132	578	329		62.4	65.4	
Sasaki *et al*[23]	2016	United States	426	28.9	297	129				
Palkovics *et al*[43]	2018	Hungary	319		114	72	133			
Dupré *et al*[8]	2018	United Kingdom	364	41.8	290	74		65.1	68.6	
Heise *et al*[22]	2017	Germany	160	21	113	47				
Shigematsu *et al*[44]	2018	Japan	396	36.4	155	93	148			
Rhu *et al*[45]	2017	South Korea	410	30.5	289	121		58.41	59.56	
Lionti *et al*[25]	2018	Italy	63	40	22	23	18			
Wang *et al*[46]	2017	China	159		130	29				
Norén *et al*[18]	2015	Sweden	3125		1109	1092	924			
Berardi *et al*[47]	2018	Belgium	62	24	11	4	47			
Cremolini *et al*[16]	2018	Italy	159	42.1	40	64	52			
McCracken *et al*[48]	2019	United States	612		388	226		55	62	
McVey *et al*[49]	2019	United States	732	26.8	397	336		59	62	
Imai *et al*[35]	2019	Japan	163	38.8	127	36				
Koch *et al*[36]	2018	Germany	30		24	5	1			
Liao *et al*[10]	2018	Taiwan	1442	58.	888	554		62.4	64.6	
Adam *et al*[50]	2011	France	186	37	106	35	41			
Wang *et al*[17]	2019	China	1508		557	593	358			

De Haas *et al*[24]: Transverse colon grouped with left rather than right sided primaries; Yamashita *et al*[26]: Data for study and validation sets respectively, midgut *vs* hindgut not left *vs* right. L: Left; R: Right.

undergoing hepatic resection for CRCLM, that patients with *l*-CRCLM had a higher 5-year PFS (18% *vs* 16%, $P = 0.009$). No papers give significant evidence to the contrary and only one study with 63 patients showing no significant difference[25].

Table 2 Overall survival data

Ref.	Year	Median OS (mo)				5 Year OS (%)				OS Hazard ratio			
		L	R	Rectum	*P* value	L	R	Rectum	*P* value	L	R	Rectum	*P* value
Zhou *et al*[27]	2017	35	33	32		27.5	23.1	23		0.85	1	1.02	
Zhang *et al*[30]	2017	22	12		0.012[a]								
		14	10										
Makowiec *et al*[37]	2018					41	46						
Chafai *et al*[29]	2005					22.5	9.9		< 0.001[a]	1	1.68		< 0.001[a]
Rougier *et al*[15]	1995	8.2	4.5	7.6	< 0.001[a]								
		6.8	3.8	6.6	< 0.001[a]								
Wang *et al*[38]	2018					38.3	46.5			1	1.08		
Gu *et al*[28]	2018	40.3	29.4		0.042[a]					1	6.2		< 0.001[a]
Gasser *et al*[12]	2019	55.5	33.5	58.2						1	1.53		0.029[a]
de Haas *et al*[24]	2010					54	36	48	0.001[a]	1	1.5		
Marques *et al*[11]	2018									1	2.1		
Russolillo *et al*[21]	2019	63.3	35.7		0.002[a]					0.82	1		< 0.001[a]
Umeda *et al*[34]	2013									1.24	1	1.62	
Zheng *et al*[31]	2018	29.5	21.9		< 0.001[a]					0.5	1		< 0.001[a]
Mavros *et al*[39]	2013									1	1.09		
Viganò *et al*[20]	2014	41.3	58.3	47.4		43.1	44.7	41.3					
Connor *et al*[40]	2016									1	2.4		0.0321[a]
Eefsen *et al*[41]	2015									0.63	1	0.57	0.045[a]
Schirripa *et al*[33]	2015	57.3	35.5	61.1	0.017[a]					1	1.59	0.95	0.017[a]
Loosen *et al*[42]	2018									1	2.32		
Amikura *et al*[32]	2018					56.1	48.4			1	1.287		
Yamashita *et al*[26]	2018					52	32		< 0.0001[a]	1	2.04		< 0.0001[a]
						78	55		0.003[a]	1	1.9		0.0009[a]
Dulundu *et al*[19]	2017	30.43	46.38	40.86		52.5	54.1	59					
Creasy *et al*[9]	2016	62.4	43.2			50.4	38.5		0.028[a]	1	1.22		0.028[a]
Sasaki *et al*[23]	2016	55.3	44.1		0.033[a]	53.7	41.5			0.76	1		0.033[a]
Palkovics *et al*[43]	2018	39	36	40									
Dupré *et al*[8]	2018	45.3	34.6		0.035[a]	37.5	25.4		0.010[a]	1	1.429		0.036[a]
Shigematsu *et al*[44]	2018									0.67	1	0.63	
Rhu *et al*[45]	2017									0.862	1		
Wang *et al*[46]	2017									0.75	1		
Norén *et al*[18]	2015	61	57	48	0.02[a]	45.8	44.5	42.6	0.02[a]	0.75	1	0.73	< 0.001[a]
Cremolini *et al*[16]	2018									0.96	1		
McCracken *et al*[48]	2019	75.6	54		< 0.001[a]					1	1.6		0.001[a]
McVey *et al*[49]	2019	43	44.2							1.108	1		
		79.4	64.6		0.037[a]					0.629	0.788		0.024[a]
Imai *et al*[35]	2019					55.5	52.3			1	3.44		0.021[a]
Koch *et al*[36]	2018									0.66	1		
Liao *et al*[10]	2018					75.2	61.7		0.005[a]				
Adam *et al*[50]	2011					31	0	36	0.003[a]	1	2.2		0.003[a]
Wang *et al*[17]	2019					40.1	24.6		< 0.001[a]	1	1.75		< 0.001[a]

Zhang *et al*[30]: Top group: Overall population, bottom row: Patients who did not receive chemotherapy; Rougier *et al*[15]: Resection subgroup data presented in row above non-resection subgroup data; Yamashita *et al*[26]: Data for study set displayed above data for validation set; McVey *et al*[49]: R1 Resection data displayed over R0 Resection data. OS: Overall Survival.

[a]$P < 0.05$. L: Left; R: Right.

DISCUSSION

The data summarised in this systematic review appear to support the suggestion that CRCLM arising from right-sided colorectal primary tumours are associated with

Table 3 Disease free survival data

Ref.	Year	Median DFS (mo)				3 yr DFS (%)				DFS HR			
		L	R	Rectum	*P* value	L	R	Rectum	*P* value	L	R	Rectum	*P* value
Wang *et al*[38]	2018					22.4	29.1						
Gasser *et al*[12]	2019	12.6	9.1	9.6									
Marques *et al*[11]	2018									1	1.1		
Russolillo *et al*[21]	2019	32.7	20.8		0.002[a]								
Connor *et al*[40]										1	1.6		
Eefsen *et al*[41]										0.60	1	0.92	
Schirripa *et al*[33]	2015					12.0	10.7	12.6		1	1.23	1.04	
Amikura *et al*[32]	2018					35.4	32.3			1.09	1		
Yamashita *et al*[26]	2018					27	15		0.001[a]	1	1.71		< 0.0001[a]
						41	21		0.001[a]	1	1.48		< 0.0001[a]
Creasy *et al*[9]	2016					37	29				1.14		
Sasaki *et al*[23]	2016					20.2	28		0.001[a]				
Heise *et al*[22]	2017									0.19	1		0.001[a]
Shigematsu *et al*[44]	2018									0.85	1	0.97	
Wang *et al*[46]	2017									1.36	1		
Berardi *et al*[47]	2018									1.06	1	1.63	
Cremolini *et al*[16]	2018									0.81	1		
Imai *et al*[35]	2019	22.9	21.3										
Liao *et al*[10]	2018					70.9	66.5		0.033[a]				

Yamashita *et al*[26]: Data for study set above data for validation set, midgut *vs* hindgut, not left *vs* right; Imai *et al*[35]: 5 Year DFS% not 3-year disease free survival.

[a]$P < 0.05$. DFS: Disease free survival; L: Left; R: Right; HR: Hazard ratio.

inferior OS compared with those arising from the left-sided CRC. Specifically, 21 of the 38 studies that provided data on OS reported statistically significant inferior OS in patients with *r*-CRCLM. Liao *et al*[10] for example demonstrated in their large study of 1442 patients that patients with *l*-CRCLM had better 5-year OS, 5-year cancer-specific survival, and 5-year RFS, all with statistical significance. In 2018 Yamashita *et al*[26] similarly concluded in their cohort of 725 patients undergoing upfront hepatic resection, that there was a significant survival benefit to having *l*-CRCLM, but that this benefit was no longer evident after neoadjuvant chemotherapy. The relationship between primary site and DFS and PFS with CRCLM is less clear, though again there appears to be a trend towards improved oncological outcome in *l*-CRCLM. Explaining these variations in oncological outcome is likely to require a deeper understanding of the underlying molecular and embryological differences associated with primary tumour sidedness.

There are subtleties in regards to variable oncological outcome identified in this review that merit further discussion. For example, in terms of DFS, Sasaki *et al*[23] reported interesting findings in terms of patterns of relapse with *l*-CRCLM compared with *r*-CRCLM. Specifically, in their study patients with *l*-CRCLM exhibited a shorter disease-free interval compared with patients who had undergone treatment for *r*-CRCLM ($P = 0.01$). However, irrespective of timing of relapse, and in spite of a longer disease-free interval, when patients with *r*-CRCLM did succumb to hepatic recurrence, it was consistently found to be with more advanced disease (> 4 recurrent lesions, $P < 0.01$). As a result, the authors found significantly reduced OS and significantly reduced survival after recurrence in *r*-CRCLM, compared with *l*-CRCLM. Thus it is conceivable, for reasons as yet unclear, that the liver is able to "hold off" recurrence of hepatic metastases arising from right-sided primaries for longer, but also that when this does finally occur, it is a more aggressive pattern of progression, leading to the paradoxical observation in some studies of seemingly favourable disease free interval, but ultimately inferior OS with *r*-CRCLM.

Previously the suggestion has been made that the typically more indolent course of presentation of right CRC, might in part be responsible for inferior outcome with resulting liver metastases[10]. The notion here is that delayed diagnosis of primary tumour results in an increased risk of developing synchronous metastases which are then incurable[51]. This would mean potentially fewer curative-intent resections offered

Table 4 Progression free survival data

Ref.	Year	Median PFS (mo)				5 yr PFS (%)				PFS HR			
		L	R	Rectum	*P* value	L	R	Rectum	*P* value	L	R	Rectum	*P* value
Zhou *et al*[27]	2017					12.5	7.1	11.5	0.012[a]	0.67	1	0.85	0.012[a]
de Haas *et al*[24]	2010					18	16	7	0.009[a]				
Zheng *et al*[31]	2018	9.2	7.3		0.002[a]					0.75	1		0.002[a]
Lionti *et al*[25]	2018									0.5	1	1	
Liao *et al*[10]	2018					70.9	66.5		0.033[a]				

[a]*P* < 0.05. PFS: Progression free survival; L: Left; R: Right; HR: Hazard ratio.

to these patients, resulting in observed abbreviated survival. However, in this study we have also shown that there is evidence that PTL also has prognostic impact in patients with unresectable disease from the outset. For example, Zhou *et al*[27] reported on outcomes in 295 patients with unresectable CRCLM undergoing palliative radio-frequency ablation, and found similar rates of OS, but that the PFS was significantly better in patients with *l*-CRCLM (HR: 0.67, *P* = 0.012). Gu *et al*[28] also reported outcomes following palliative-intent radio-frequency ablation in patients with CRCLM, finding that patients with *l*-CRCLM had a significantly lower risk of recurrence outside of the ablation zone, with increased OS of 40.3 mo compared with 29.4 mo in *r*-CRCLM(*P* = 0.042). Multivariate analysis confirmed a HR of 6.2 (*P* = 0.001) for *r*-CRCLM predicting OS. This data is further supported by findings reported by Chafai *et al*[29] in 2005, who studied patients with unresected synchronous liver metastases after resection of the primary tumour. They found a significantly shorter survival in palliative patients who had *r*-CRCLM compared with *l*-CRCLM (2 years survival 9.9% *vs* 22.2%, HR: 1.5 *P* < 0.001). In circumstances where palliative/debulking surgery is offered, differences continue to persist for l-CRCLM versus r-CRCLM. For example, in 2017 Zhang *et al*[30] found that hepatic palliative resection prolonged median OS by 8 mo in patients with *l*-CRCLM (palliative resection *vs* no resection: 22 mo *vs* 14 mo, *P* = 0.009); however, by comparison no such improvement in OS was observed for patients with *r*-CRCLM undergoing palliative resection (12 mo *vs* 10 mo, *P* = 0.910).

With regards to defining putative mechanistic explanations for these differences, a number of factors should be considered. Firstly, there is considerable evidence that right sided CRCs are significantly more likely to harbor negative prognostic features; they tend to present at a more advanced stage, often in older patients, with a greater chance of synchronous metastatic disease, are more likely to carry unfavourable genetic mutation(s), and show poor differentiation[18,21,27,31,52]. It could therefore follow that patients with right sided CRC simply present with more advanced and aggressive disease from the outset. This however was not a uniform finding across the studies included in this review. For example, Creasy *et al*[9] found no such differences between right sided versus left sided CRC in terms of proportion of patients with the largest metastasis > 5 cm, proportion of patients with multiple metastases, or the proportion of patients with extra-hepatic disease. In spite of this relative equipoise, the authors reported significantly improved OS in patients with *l*-CRCLM and suggest that unique differences based on sidedness are likely to exist that extend beyond the aforementioned conventionally accepted differences.

From this perspective a number of mechanisms have emerged that could play a role in contributing to the inferior oncological outcome observed in patients with *r*-CRCLM. These broadly can be considered as: (1) Molecular differences; (2) Histopathological differences; (3) Therapeutic sensitivity differences; and (4) Embryological differences.

Molecular differences

There are well-established molecular differences between right- and left-sided CRC with the former more often exhibiting *KRAS* and/or *BRAF* mutation[12,33,34,53]. RAS mutations have consistently been found to be associated with more aggressive tumour biology and are identified in up to 45% of patients with metastatic CRC. For example, the studies published by Amikura *et al*[32] and Shindoh *et al*[54] both demonstrate that RAS mutational status is associated with significantly worse survival in CRCLM (Amikura *et al*[32]: 5-year OS: 42.4% *vs* 65.3%, *P* = 0.0006; Shindoh *et al*[54]: 3-year DFS 59.9 *vs* 83.6% *P* = 0.016). Of note, it has also been reported that among patients put forward for curative intent resection of CRCLMs, the incidence of RAS

mutation is only around 10%-15%, indicating that underlying tumour biology, seemingly inseparably linked to PTL, exerts additional prognostic relevance as it appears to indirectly influence surgical candidacy[55]. Goffredo *et al*[56] evaluated outcomes in 2655 patients undergoing CRCLM resection. They observed a significant increase in likelihood of mutant KRAS with right-sided PTL, compared to left and correspondingly found reduced OS in patients with *r*-CRCLM. It is likely that additional molecular drivers are responsible for the variations seen according to PTL, and RAS/BRAF likely account for only part of the molecular landscape especially since only a limited proportion of these cases are put forward for resection[54]. This notion is supported by Huang *et al*[57] who found no significant association between KRAS/BRAF mutational status and prognosis in patients presenting with metachronous CRCLM.

The role of mismatch repair (MMR) status and microsatellite instability (MSI) in the context of PTL seems less certain. Right sided CRC is more frequently associated with deficient MMR and MSI[7,56,58]. These tumours tend to be typified by poor differentiation, mucinous features and lymphocytic invasion. Evidence supports the suggestion that MSI is associated with improved oncological outcome[59,60]. However, this is at odds with the findings of the present review, where *r*-CRCLM appears to have shortened survival. This may reflect fundamental differences in MMR status according to tumour stage. For example, Jernvall *et al*[61] found MSI to be a more common finding in right sided Stage II CRC, but this was less frequently observed in stage IV disease. In this review molecular data were only available from a limited number of publications and subdivision of molecular phenotype according to PTL has not been provided in most cases. Hence, we are not able to draw any more definitive conclusions on the precise interplay between molecular factors and PTL. Considering these limitations, The Cancer Genome Atlas Network sought to evaluate a broader panel of genetic mutations and defined cases as "hypermutated" where a mutation rate of > $12/10^6$ bases was found. Out of 276 samples analysed, the majority of hypermutated cases were right sided primary tumours. The group suggest that hypermutated phenotype is a significant negative prognostic feature, and this may in part account for inferior survival with *r*-CRCLM, as noted in the present review[62].

Histological differences

Several investigators have evaluated tumour histopathological features in order to determine if the difference in sidedness outcomes and tumour aggressiveness can be explained by one or more of these. Desmoplastic growth behaviour, presence of poorly differentiated clusters and tumour budding have all been considered[25,41,63]. Strong evidence, however, relates to the prevalence of mucinous elements. Viganò *et al*[20] and Russolillo *et al*[21] have both demonstrated mucinous adenocarcinoma to be more prevalent in right-sided CRC ($P = 0.002$ and $P = 0.001$, respectively). Viganò *et al*[20] reported significantly shortened OS with *r*-CRCLM versus *l*-CRCLM. They observed that, when compared with non-mucinous carcinoma, mucinous carcinoma has a higher KRAS mutation rate 61.8% *vs* 36.4%; $P = 0.037$) and lower chemotherapy response rate (63.9% *vs* 85.2%; $P = 0.006$). Specifically, Viganò *et al*[20] reported lower 5-year OS (33.2% *vs* 55.2%; $P = 0.010$) and DFS (32.5% *vs* 49.3%; $P = 0.037$) for mucinous tumours undergoing hepatic resection. One can extrapolate from these observations that inferior survival and right sided PTL are linked by an increased tendency for mucinous histology.

Therapeutic sensitivity differences

There are also suggestions in the literature that chemosensitivity is important in predicting survival, and that there may be a differing chemosensitivity profile according to PTL. In their meta-analysis of 16 first-line trials evaluating the efficacy of chemotherapy alone *vs* chemotherapy with targeted biologics in patients with unresectable metastatic CRC, You *et al*[64] found survival of patients with right sided CRC was inferior to those with left in patients receiving chemotherapy alone, implying that right-sided tumours overall are less chemosensitive. This finding is supported by Yamashita *et al*[26] found that *r*-CRCLM were independently associated with "minor pathological response" (defined as cancer cells accounting for ≥ 50% of residual cells), and were thus less sensitive to chemotherapy with worse RFS and OS. Interestingly, Marques *et al*[11] found that when selecting patients for CRCLM resection based on chemosensitivity, the survival disadvantage seen with *r*-CRCLM was eliminated. This suggests fundamental differences in tumour biology with the less chemosensitive phenotype more frequently seen with right-sided PTL and in turn associated with poorer survival.

This difference is maintained after the addition of well-established antiangiogenic biologics. You *et al*[64] found inferior survival in patients with *r*-CRCLM receiving chemotherapy and bevacizumab compared with *l*-CRCLM. Zheng *et al*[31] studied the

effect of cetuximab as an addition to chemotherapy in KRAS wild-type patients with initially unresectable hepatic metastases. They found a survival benefit to cetuximab in patients with both *r*-CRCLM and *l*-CRCLM, but importantly noted that this effect was more substantial in the latter, with higher rates of effective tumour downstaging and extended OS.

Embryological differences

In terms of embryology, Yamashita *et al*[26] suggest that the mid-gut embryological origin of the right colon may be responsible for the variable responsiveness of *r*-CRCLM to chemotherapy and differing oncological outcomes. Specifically, in their study of outcomes in 725 patients, they found reduced responsiveness to chemotherapy, reduced RFS and reduced OS in patients with *r*-CRCLM. The authors reported that this difference was maintained irrespective of RAS mutational status, which is considered to be one of the key oncogenic differences between right- and left-sided colon cancers. It is possible however, that other factors centered around the distinct development of these regions of gut, including unique lymphatic and venous drainage basins, and exposures to unique types of bacterial flora, could be contributing to oncological variability. This is an area that requires further research.

Limitations

As a systematic review this paper has some inherent limitations, it is restricted by the quality of the literature available. However, all included papers were graded using the SIGN criteria with small studies excluded to mitigate this. Care was taken to perform a complete literature search, but studies and some work in progress may have been missed. Larger studies and well as future meta-analysis will be necessary to more clearly establish this trend and may provide a deeper understanding of the mechanisms at play.

In conclusion, the present review provides compelling data to support the notion that PTL significantly influences oncological outcome in patients with CRCLM. Overall, the data presented indicate that patients with *r*-CRCLM appear to have truncated overall, disease-free and progression-free survival. Some of these differences are likely to be accounted for by molecular heterogeneity, but other factors such as embryological origin and colonic microbiotal composition are areas that have received comparatively less attention in terms of research, and these may represent promising avenues to explore in the future. With the understanding that PTL could have prognostic relevance, comes the need to adjust treatment pipelines for patients accordingly. For example, patients with right-sided CRC may require abbreviated intervals between surveillance scans and tumour-marker assessment after primary tumour resection. In addition, given their more aggressive pattern of recurrence after hepatic resection/treatment, patients with *r*-CRCLM may benefit from a more radical first-line treatment of hepatic metastases. For example, the role of non-anatomical resection and the use of locally-ablative techniques in *r*-CRCLM may need more careful consideration.

ARTICLE HIGHLIGHTS

Research background

Colorectal cancer (CRC) is the third most common cause of cancer related death with liver being the most common metastatic site. It has been long suggested that left and right sided primary tumours exhibit different behaviour but relatively little has been written about how this relates specifically to outcomes in colorectal cancer with liver metastases (CRCLM).

Research motivation

To improve current understanding regarding the impact of PTL on CRCLM given the relative paucity of information in this area. This in turn could have a significant impact on patient morbidity and mortality.

Research objectives

To ascertain whether there is a significant difference in oncological outcome in patients with CRCLM depending on PTL and to present some hypotheses that may explain any differences found. This systematic review demonstrates a significant difference in outcomes based on PTL with inferior oncological outcome for patients with right-sided CRC. . Further work is needed to better characterise the mechanisms responsible for this variation in order to inform clinical decision making.

Research methods

A systematic review of Medline, Cochrane and Embase using the Terms "The medical subject heading terms and key words used are as follows: "Colon" or "rectal cancer", "liver metastasis" or "liver metastases" or "hepatic metastasis" or "hepatic metastases" and "left" and "Right".

This search was combined with a bibliographic search to find the relevant publications and extract data from these papers. The methodology was based around the Preferred Reporting Items for Systematic Reviews and Meta-Analyses' recommendations for systematic reviews

Research results

Twenty-one studies with a total of 18203 patients showed a statistically significant trend of improved overall survival in patient with left sided primary tumours undergoing treatment for colorectal cancer liver metastases (*l*-CRCLM). Four studies including 3013 patients showed improved disease free survival (DFS) in *l*-CRCLM. Only five publications provided data on progression free survival (PFS). These studies including 2805 patients showed significantly improved PFS in *l*-CRCLM *vs* *r*-CRCLM. The findings of this review are congruent with the accepted premise of superior survival in left sided colorectal cancer, and uniquely show that this remains true in the context of metastatic liver disease. We highlight a number of factors that may contribute to this, including KRAS/BRAF mutational status, presence of mucinous elements, and impaired chemosensitivy –all which are shown to be associated with right-sided PTL. The exact interplay between these known factors, PTL, and the emerging new mutations and molecular markers is yet to be determined and work needs to be done to determine the importance of PTL within the conglomeration.

Research conclusions

The findings of this review indicate that PTL may have a role as an independent prognostic factor when determining treatment and disease surveillance strategies specifically in colorectal cancer that has metastasised to the liver. We find improved survival for both resected and unresectable *l*-CRCLM as well as a maintained trend after addition of biologics to established chemotherapy regimens. Hepatic recurrence after treatment of CRCLM appears to occur more aggressively with right-sided CRC, conferring significantly reduced survival. Explaining these variations in oncological outcome requires a deeper understanding of the underlying molecular and embryological differences associated with primary tumour sidedness. Microsatellite instability, interestingly, whilst more common in right-sided tumours, has been shown to be independently associated with improved survival – a finding somewhat incongruent with the overall picture of inferior survival in *r*-CRCLM. This suggests alternative mechanisms beyond MMR and microsatellite instability are likely to be involved. KRAS and BRAF mutational status, mucinous adenocarcinoma, and impaired chemosensitivity are all known to be significantly associated with right-sided CRC, and we show here that this association and the accompanying inferior survival persists in *r*-CRCLM. A better understanding of the role of PTL in the oncological outcomes of metastatic CRC may allow for improved risk stratification and redesigned patient pathways.

Research perspectives

There is a considerable amount of data available on the oncological outcomes of patients undergoing liver resection for CRCLM, as related to PTL. This shows with convincing evidence that outcomes are superior for patients with *l*-CRCLM. Future research should be focused on gathering associated molecular and genetic data as related to PTL to better understand the tumour biology of right-sided CRC. This may allow the determination of ideal molecular markers, both for risk stratification/prognostication, and that may be used as potential therapeutic targets.

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