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OPINION REVIEW

Advances and controversies in the management of early stage non-small cell lung cancer

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Abstract

Complete resection continues to be the gold standard for the treatment of early-stage lung cancer. The landmark Lung Cancer Study Group trial in 1995 estab-

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lished lobectomy as the minimum intervention necessary for the management of early-stage non-small cell lung cancer, as it was associated with lower recurrence and metastasis rates than sublobar resection and lower postoperative morbidity and mortality than pneumonectomy. There is a growing tendency to perform sublobar resection in selected cases, as, depending on factors such as tumor size, histologic subtype, lymph node involvement, and resection margins, it can produce similar oncological results to lobectomy. Alternative treatments such as stereotactic body radiotherapy and radiofrequency ablation can also produce good outcomes in inoperable patients or patients who refuse surgery.

Key Words: Video-assisted thoracoscopic surgery; Sublobar resection; Radiofrequency ablation; Stereotactic radiosurgery; Early stage; Lung cancer

Core Tip: Complete resection continues to be the gold standard for the treatment of early-stage lung cancer. Lobectomy remains the gold standard for the treatment of early-stage non-small cell lung cancer, but there is a growing tendency to perform sublobar resection in selected cases. Alternative treatments such as stereotactic body radiotherapy and radiofrequency ablation can also produce good outcomes in inoperable patients or patients who refuse surgery.

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INTRODUCTION

Complete resection continues to be the gold standard for the treatment of early-stage lung cancer. The landmark Lung Cancer Study Group trial in 1995 established lobectomy as the minimum intervention necessary for the management of early-stage non-small cell lung cancer (NSCLC), as it was associated with lower recurrence and metastasis rates than sublobar resection and lower postoperative morbidity and mortality than pneumonectomy[1]. The development of lung-sparing techniques (*e.g.*, sleeve resection with vascular and/or bronchial reconstruction) has reduced the number of pneumonectomies performed and with this the risk of adverse outcomes, as the proportion of pneumonectomies is a quality indicator in thoracic surgery[2].

While lobectomy remains the gold standard for the treatment of early-stage NSCLC, there is a growing tendency to perform sublobar resection in selected cases, as, depending on factors such as tumor size, histologic subtype, lymph node involvement, and resection margins, it can produce similar oncological results to lobectomy[3]. Two randomized clinical trials comparing lobectomy and sublobar resection are currently underway: The United States Cancer and Leukemia Group B trial (CALGB 140503)[4] and the Japanese JCOG0802/WJOG4607L trial[5]. The results so far have shown no significant differences in postoperative morbidity or mortality, but as discussed in greater detail below, data on survival and pulmonary function are pending.

The use of minimally invasive techniques for the surgical treatment of early-stage NSCLC has increased in recent years. Video-assisted thoracoscopic surgery (VATS) is the current procedure of choice for most resections in this setting. A recent nationwide cohort study conducted in Spain reported that over 50% of recent anatomic lung resections had been performed by VATS[6]. The main advantages of VATS compared with open surgery are decreased postoperative pain, fewer postoperative complications, and in some cases even, better oncological outcomes. There are, however, substantial geographic variations in the use of VATS.

Advances in VATS techniques and the design of specific surgical material have led to a progressive reduction in the number of incisions required. Most thoracic surgeons now use between one and three incisions and describe similar oncological results[6,7]. Subxiphoid VATS is another minimally invasive technique associated with good outcomes when performed by teams with extensive experience in VATS; it has been linked to a lower incidence of postoperative neuropathic pain[8].

The increasing adoption of VATS has favored its use in more locoregionally advanced lung cancers. Data from large series of angio-bronchoplastic or extended lung resections performed by experienced thoracic surgeons show similar outcomes to thoracotomy[9].

Good outcomes have also been described with robotic-assisted thoracoscopic surgery in the setting of anatomic resections, although the cost-effectiveness of the technique is not so clear[10].

As we discuss below, alternative treatments such as stereotactic body radiotherapy (SBRT) and radiofrequency ablation (RFA) can also produce good outcomes in inoperable patients or patients who refuse surgery.

ROLE OF SUBLOBAR RESECTION IN LUNG CANCER

Anatomic sublobar resections have produced comparable oncological results to lobectomy in the treatment of tumors < 2 cm without nodal involvement or distant metastasis[11]. These favorable results have led to an increased use of segmentectomy, which, depending on tumor stage and resection margins, can produce similar oncological results to lobectomy in selected patients[12].

Anatomic segmentectomy is oncologically more valuable than atypical (wedge) resection in early-stage cancer as it permits the performance of hilar and mediastinal lymph node dissection[13].

Its main advantage, however, is its parenchyma-sparing effect, which results in better postoperative respiratory function than lobectomy. In view of the above, anatomic sublobar resection can be considered an appropriate treatment for patients with compromised respiratory function unable to tolerate standard lobectomy. Patients considered to be at high operative risk include patients with FEV_1 < 50% or DLCO < 50% and elderly patients with impaired lung function, pulmonary hypertension, and poor left ventricular function[14,15].

Compared with lobectomy, VATS sublobar resection has been linked to shorter hospital stays and drainage times, a lower incidence of supraventricular arrhythmia, and fewer postoperative respiratory complications[11].

In certain cases, anatomic segmentectomy involves a higher risk of air leakage when electrocautery is used for intersegmental plane dissection (as reported by several Japanese groups)[5,13]. Air leakage is not common when absorbable sutures are used, which is the case in most lung resections.

The only randomized prospective trial to compare lobectomy and sublobar resection in T1N0M0 lung cancer (the Lung Cancer Study Group trial) concluded that patients treated with sublobar resection had a higher risk of locoregional recurrence and death[1]. It should be noted, however, that these results were published in 1995 and that lung tumors are now diagnosed earlier.

Several retrospective studies published since 2000 have reported good oncological outcomes in patients with small peripheral tumors (stage I and < 2 cm) treated with segmentectomy[13,16-19].

As mentioned, the ongoing CALGB[4] and Japanese[5] trials have not detected any differences between lobectomy and sublobar resection for postoperative morbidity or mortality, but survival and pulmonary function outcomes are not yet available[4,13,20].

Thus, it remains to be determined whether segmentectomy is a valid alternative to lobectomy for the treatment of early-stage NSCLC in patients fit for both procedures[4,5].

POSTRESECTION ADJUVANT THERAPY IN NSCLC

Thirty percent of lung cancer patients have early-stage disease when diagnosed. The standard treatment is surgery, followed or not by chemotherapy with or without radiotherapy.

Data from retrospective series show that less invasive surgical procedures result in fewer complications, allowing earlier initiation of chemotherapy, but do not appear to have an impact on overall survival (OS).

Postoperative radiotherapy in stage I and II NSCLC is indicated for patients with positive margins. According to the recent results of the phase III LUNG ART trial, postoperative radiotherapy did not have any beneficial effects in patients with pathologic mediastinal involvement (N2), in addition, it induced high levels of toxicity. Chemotherapy, however, was associated with a 5.4% increase in OS at 5 years, regardless of age [hazard ratio (HR) = 0.89]. Chemotherapy is indicated for resected stage II and IIIA NSCLC[21], but its use in stage I disease is more controversial. The standard treatment is four cycles of doublet cisplatin-based chemotherapy. The only clinical trial to investigate the use of carboplatin in this setting reported negative results[22]. Survival outcomes, however, are poor, mainly because of high rates of distant recurrence. Five-year OS rates range from 73% for stage IB disease to 65% for stage IIA disease, 56% for stage IIB disease, and 41% for stage IIIA disease[23]. It is therefore important to continue to explore new treatments and prognostic and predictive biomarkers.

Attempts to improve treatment outcomes with the addition of antiangiogenics[24] or vaccine-based therapy[25] have been unsuccessful. The potential benefits of immunotherapy are being investigated, as good results have been reported for adjuvant immunotherapy in more advanced stages of disease and other types of tumor [26]. Ongoing trials include PEARLS (pembrolizumab), BR31 (durvalumab), ANVIL (nivolumab), Impower 010 (atezolizumab), and Canopy-A (canakinumab). No results, however, are available yet. Immunotherapy, both alone and combined with chemotherapy, has shown promising results in the neoadjuvant setting. Chemoimmunotherapy has significantly improved complete and major pathological responses in NSCLC (by approximately 36% and 65%, respectively) and has also led to downstaging in over 70% of patients[26,27]. It remains to be determined whether immunotherapy is more effective as a neoadjuvant or adjuvant treatment[28].

Agents targeting driver mutations are being investigated in multiple trials, but results are still pending. We do have results from the ADAURA trial, where patients with completely resected *EGFR* mutation–positive NSCLC, regardless of whether or not they had received prior chemotherapy, were randomized to receive osimertinib [a third-generation tyrosine-kinase inhibitor (TKI)] or placebo for 3 years. The progression-free survival (PFS) outcomes for patients with stage II and IIIA disease in the osimertinib group were unprecedented, with an HR for disease recurrence or death of 0.17. In addition, the benefits were observed in all the subgroup analyses. The adverse events were to be expected based on the experience with this drug. Osimertinib was also associated with a reduction in brain recurrences (HR = 0.18)[29]. These results were sufficient for the United States Food and Drug Administration to approve osimertinib as an adjuvant treatment for NSCLC with *EGFR* mutations. Recent results from another trial showed that icotinib, a first-generation TKI, improved PFS (HR = 0.36) in patients with resected stages II and IIA disease; results on OS have not been published yet[30]. Nonetheless, in the CTONG trial of adjuvant treatment with gefitinib, the improvement observed for PFS was not carried over to OS, reflecting previous findings for other targeted therapies. It remains to be seen whether osimertinib will achieve a survival benefit in the ADAURA trial.

Little has been reported on the use of biomarkers in this setting, as they were not a requirement in most of the trials conducted to date. Thus, the potential values of *BRCA1* and of *ERCC1* and thymidylate synthase were not validated in the respective SCAT and ITACA trials. Contradictory results have been reported for the prognostic value of PDL-1 expression and tumor mutational burden[31-33]. Nonetheless, next-generation sequencing is a promising strategy for the detection of residual disease after surgery[34,35]. A recent meta-analysis showed that residual molecular disease detected by circulating tumor DNA analysis after complete resection was associated with a higher risk of recurrence and death.

Despite the available evidence, treatment should always be individualized, with careful assessment of risks and benefits, particularly in the current scenario of COVID-19[36].

SBRT IN EARLY-STAGE LUNG CANCER

SBRT is a high-precision technique that delivers high doses of radiation over a short period of time[37]. Conceptually derived from cranial stereotactic radiosurgery, it is

now used in multiple anatomic locations. It is the treatment of choice for early-stage lung cancer in medically inoperable patients or patients who refuse surgery, with a 5-year local control rate of 90%[38]. It improves survival in older patients and reduces the number of untreated patients. When SBRT is not feasible, hypofractionated radiotherapy is preferred to conventionally fractionated schedules[39]. Acute toxicity is rare in SBRT, and includes mild fatigue 1-2 wk after treatment; quality of life is rarely affected[40]. The risk of severe toxicity is low[41], and the most common adverse effect is decreased lung capacity. SBRT can be highly toxic in patients with a history of interstitial lung disease and its use should be assessed by a multidisciplinary committee. Late adverse effects include pain, rib fractures, dyspnea, and ventricular tachycardia[38]. Other effects are esophagitis, epithelitis, and brachial plexopathy. Complications are largely influenced by tumor location and size, radiation dose and target volume[42]. Pathological confirmation is not always possible, and some authors have suggested that up to 16% of lung nodules may be benign[4].

SBRT has certain technical characteristics that need to be taken into account when planning and administering treatment. Four-dimensional computed tomography (CT) is recommended for preoperative simulation, and multiple beams or arcs should be used for planning purposes as they help limit toxicity[43].

Dose schedules for peripheral tumors vary, but mostly consist of 3-8 fractions of 7.5-20 Gy each; results for a dose of 54 Gy in 3 fractions include a 3-year local control rate of 91%, a 3-year disseminated failure rate of 22%[44], a 5-year local control rate of 80%, and a 5-year local control rate of 31%[41]. A phase II trial comparing 30 Gy in 1 fraction and 60 Gy in 3 fractions showed 2-year survival rates of 71% and 61%, respectively, with no differences in toxicity[45]. On comparing 34 Gy in 1 fraction and 48 Gy in 4 fractions, Nagata *et al*[46] found OS rates of 61% and 78%, respectively, and no differences in survival, primary tumor control, or toxicity. In their meta-analysis of 34 observational studies involving 2597 patients, Zhang *et al*[47] determined that the most beneficial dose regimens were those that achieved a biologically equivalent dose of 83.3-146 Gy[47].

Centrally located tumors are tumors located within 2 cm, in any direction, of a critical mediastinal structure, such as the bronchial tree, esophagus, heart, brachial plexus, major vessels, spinal cord, phrenic nerve, and recurrent laryngeal nerve. SBRT is not suitable for ultracentral tumors, but hypofractionated schedules consisting of 6-15 fractions could be considered[48]. Risk-adapted schedules have achieved high local control rates and limited toxicity. Evidence to date shows a 5-year OS rate of 50% and a local control rate of 93%[49,50]. A systematic review of SBRT efficacy and toxicity in centrally located NSCLC showed similar local control and survival rates to those achieved in peripheral tumors.

Three randomized clinical trials have compared SBRT and surgery, although they had problems with accrual. A pooled analysis of the STARS and ROSEL trials showed comparable 3-year recurrence-free survival. Results from the ACOSOG Z4099 trial have not been reported. In the RTOG 0813 trial, 100 medically inoperable patients with central tumors were treated with 50-60 Gy in 5 fractions on alternating days. This resulted in 2-year local control, OS, and PFS rates of 88%, 70%, and 53%, respectively; 15 patients experienced grade 3 or higher toxicity (grade 3, 10 patients; grade 4, one patient; and grade 5, four patients). The standard treatment for patients with operable tumors is surgery, lobectomy, and mediastinal lymph node dissection. The RTOG 0236 [41] and 0915[47] trials showed a 3-year OS rate of 56% over a median follow-up of 4 years and a 5-year OS rate of 40%. The local control and 3-year survival rates were 87.3% and 59.9%, respectively. High recurrence rates, however, were observed in the SBRT group during follow-up[51,52]. Results from the VALOR, SABRTooth, RTOG 3502, and STABLE-MATES trials are pending (Table 1).

When used to treat multiple synchronous tumors *vs* solitary tumors, SBRT offers similar local control and toxicity rates and worse survival rates[53]. The role of SBRT is being investigated in T3-4N0M0 tumors with schedules of 8-10 Gy per fraction in 8-10 fractions. Two-year local control rates of 68%-73.2% have been described[54-56].

A recent study demonstrated that SBRT after contralateral pneumonectomy was safe. Arifin *et al*[57] analyzed 59 studies with a mean follow-up of 25.4 mo and found a mean 1-year OS rate of 80.6%, a 2-year local control rate of 89.4%, and a grade ≥ 3 rate of 13.2%.

RFA IN EARLY-STAGE NSCLC

RFA is a minimally invasive CT-guided procedure originally approved for use in liver

Table 1 Studies analyzing surgery and stereotactic body radiotherapy in non-small cell lung cancer

Ref.	Type	Surgery-RT, No.	Surgery-RT, Local failure	Surgery-RT, PFS	Surgery-RT, OS	Surgery-RT, Toxicity	LoE
Grills *et al* [74], 2010	R	69 wedge resection; 58 SBRT; Unfit for lobectomy	20%-4% (P = 0.07)	65% *vs* 77% (P = 0.37)	87% *vs* 72% (P = 0.01)	Readmission 10%; Pneumonitis 2%; Fracture 11%	3
Varlotto *et al* [75], 2013	R	48 sublobar resection +132 lobectomy; 137 SBRT	At 5 yr 18.8% lobectomy *vs* SBRT 12.2% (P = 0.382); Resection 7.1%	No differences (P = 0.378)	At 5 yr lobectomy *vs* SBRT 33.7%; Resection 86.3% (P = 0.04, P = 0.003)		3
Verstegen *et al*[76], 2013	R	64 VATS; 64 SBRT; 54% inoperable	At 3 yr 3.1% *vs* 1.6% (P = 0.04)	79.7% *vs* 75%	76.9% *vs* 90.8% (P = 0.83)	23.4% *vs* 6.3% G ≥ 3 (P = 0.03)	3
Matsuo *et al* [77], 2014	R	53 sublobar resection; 53 SBRT	At 5 yr 14.1% *vs* 28.3% (P = 0.059)		55.6% *vs* 40.4% (P = 0.124)		3
Zheng *et al* [78], 2014	MA	11921; 7071 surgery; 4850 SBRT	At 1 yr 93% lobectomy *vs* 91.5% sublobar resection *vs* 96.3% SBRT. At 3 yr 85% *vs* 78.4% *vs* 87.8%. At 5 yr 80% *vs* 63.4% *vs* 83.9% (P = 0.45)	At 1 yr 93.5% lobectomy *vs* 90.3% sublobar resection *vs* 87.1% SBRT. At 3 yr 82.9% *vs* 82.1% *vs* 65.8%. At 5 yr 74.8% *vs* 71.2% *vs* 65.8% (P = 0.46)	At 1 yr 92.5% lobectomy *vs* 93.2% sublobar resection *vs* 83.4% SBRT. At 3 yr 77.9% *vs* 80.7% *vs* 56.6%. At 5 yr 66.1% *vs* 71.7% *vs* 41.2% HR = 0.52, 95%CI: 0.2-1.36 for lobectomy and HR = 0.49, 95%CI: 0.19-1.3 for sublobar resection		1
Yu *et al*[79], 2015	R	1078; 711 surgery; 367 SBRT			At 2 yr 77.7% *vs* 59.9% (P = 0.01)	Acute 54.9% *vs* 7.9% (P < 0.001). Chronic 73.9% *vs* 69.7% P = 0.31)	3
Rosen *et al* [80], 2016	R	1781 lobectomy; 1781 SBRT			At 5 yr 59% *vs* 29%; 58% *vs* 40% for patients who refused surgery (P = 0.010)		3
Ma *et al*[81], 2016 (adjusted for operable patients)	MA	6969; 3436 VATS; 4433 SBRT		No differences (P = 0.378)	No differences HR = 2.02, 95%CI: 0.45-3.07 (P = 0.36)		2
Deng *et al* [82], 2017	MA	13598	No differences (P = 0.453)		At 3 yr 68.1% *vs* 47.7% (P < 0.001)		1
Grills *et al* [74], 2010	P. III	222 Lobectomy; 254 SBRT	At 5 yr 5% *vs* 8% (P = 0.388)	At 5 yr 72% *vs* 53% (P = 0.018)	At 5 yr 78% *vs* 61% (P = 0.006)		1
Ackerson *et al* [83], 2018	R	151 surgery; 70 SBRT	At 3 yr 10% *vs* 15% (P = 0.71)	42% *vs* 29% (P = 0.004)	At 3 yr 63% *vs* 35% (P < 0.001)	23%-17%	3
Tamura *et al* [84], 2019	R	141 surgery; 106 SBRT	Higher for SBRT (P = 0.0082)	At 5 yr 69.7%-50.2% (P = 0.036)	At 5 yr 69.7% *vs* 50.2% (P = 0.036)	8.6% surgery; SBRT G ≥ 2 7.5%	3

G: Grade; LoE: Level of evidence; MA: Meta-analysis; P: Phase; OS: Overall survival; RT: Radiotherapy; VATS: Video-assisted thoracoscopic surgery.

tumors. It is a percutaneous technique that consists of applying an alternating current (420-500 kHz) to the tumor tissue, resulting in high temperatures (> 70 °C) that cause tissue necrosis and protein denaturation[58].

Because air is a poor conductor of electricity and a good thermal insulator, the lung is theoretically an ideal site for the application of RFA as the surrounding parenchyma is barely affected[59]. The use of RFA to treat lung tumors was first described by Dupuy *et al*[60] in 2000.

The main advantages of RFA over surgery are that it is minimally invasive (percutaneous technique performed with local anesthesia), can be administered on an outpatient basis or under 24-h hospitalization, and does not require thoracotomy[59].

The use of RFA is limited to the treatment of lesions < 3 cm located in the outer two-thirds of the lung parenchyma. Tumor size affects the homogeneity of the temperature distribution within the lesion. Tumors > 3 cm require the use of several overlapping electrical fields to achieve a high enough temperature, and this increases the risk of complications. As with surgery, a margin of healthy parenchymal tissue must be included in the radiofrequency field, but this is difficult to achieve because of the

thermal insulation effect mentioned above[61-63].

Central lesions carry a higher risk of complications due to their proximity to the bronchial tree, esophagus, and heart. RFA may be less effective when applied to tumors located close to blood vessels with a diameter > 0.3 cm due to what is known as a "heat sink" effect (a cooling effect caused by the constant renewal of blood within the vessel)[59].

The main adverse effects associated with RFA are pneumothorax [the most common complication (11%-67%) following removal of the electrode from the parenchyma], pleural effusion (related to the increase in pleural temperature), hemoptysis, and more rarely, infections, bronchial fistula, and nerve or cardiac injuries.

In a recent meta-analysis comparing RFA and sublobar resection, Chen *et al*[59] analyzed four retrospective studies involving 309 patients: 155 treated with RFA and 154 with sublobar resection. The patients who underwent sublobar resection had significantly higher 1- and 3-year OS and PFS rates (97% *vs* 91% for 1-year OS, 67% *vs* 52% for 3-year OS, 91% *vs* 81% for 1-year PFS, and 67% *vs* 48% for 3-year PFS). Patients in the RFA group had more complications, but they were milder than those seen in the sublobar resection group.

In their prospective phase II trial of 42 patients with inoperable early-stage lung cancer, Palussière *et al*[64] concluded that RFA was a well-tolerated technique with 1- and 3-year local control rates of 84.38% and 81.25%, respectively, and comparable OS rates to those achieved with SBRT. Good tolerability has also been described by other authors[65], including Li *et al*[61] in their meta-analysis of 1989 patients.

Few studies have compared local treatments (RFA and SBRT), and the little evidence that exists is based on unbalanced, retrospective data. Randomized prospective studies are needed. Authors who have compared RFA and SBRT, however, agree that SBRT should be the technique of choice for inoperable early-stage cancer because of its favorable safety profile and greater survival benefits. RFA, in turn, should be reserved for small tumors not located near vessels or mediastinal structures[66-68].

At the molecular level, hypoxia-inducible factor-1α has been proposed as an independent prognostic marker in the setting of RFA, as high levels have been linked to an increased risk of mortality[69].

In conclusion, RFA may be useful for treating inoperable early-stage lung cancer, in particular tumors < 3 cm located far from the mediastinum and vessels with a diameter > 0.3 cm[70,71]. The poorer outcomes reported for RFA compared with sublobar resection may be due to the lack of randomized, prospective studies comparing the two treatments, as studies to date have included patients who are unfit for surgery, that is older, more frail patients with more comorbidities and as a result a worse prognosis[72,73].

CONCLUSION

Complete resection continues to be the gold standard for the treatment of early-stage lung cancer. Lobectomy remains the gold standard for the treatment of early-stage NSCLC, but there is a growing tendency to perform sublobar resection in selected cases. Alternative treatments such as SBRT and RFA can also produce good outcomes in inoperable patients or patients who refuse surgery.

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REVIEW

Liver regeneration biology: Implications for liver tumour therapies

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Abstract

The liver has remarkable regenerative potential, with the capacity to regenerate after 75% hepatectomy in humans and up to 90% hepatectomy in some rodent models, enabling it to meet the challenge of diverse injury types, including physical trauma, infection, inflammatory processes, direct toxicity, and immunological insults. Current understanding of liver regeneration is based largely on animal research, historically in large animals, and more recently in rodents and zebrafish, which provide powerful genetic manipulation experimental tools. Whilst immensely valuable, these models have limitations in extrapolation to the human situation. In vitro models have evolved from 2-dimensional culture to complex 3 dimensional organoids, but also have shortcomings in replicating the complex hepatic micro-anatomical and physiological milieu. The process of liver regeneration is only partially understood and characterized by layers of complexity. Liver regeneration is triggered and controlled by a multitude of mitogens acting in autocrine, paracrine, and endocrine ways, with much redundancy and cross-talk between biochemical pathways. The regenerative response is variable, involving both hypertrophy and true proliferative hyperplasia, which is itself variable, including both cellular phenotypic fidelity and cellular trans-differentiation, according to the type of injury. Complex interactions occur between parenchymal and non-parenchymal cells, and regeneration is affected by the status of the liver parenchyma, with differences between healthy and diseased liver. Finally, the process of termination of liver regeneration is even less well understood than its triggers. The complexity of liver regeneration biology combined with limited understanding has restricted specific clinical interventions to enhance liver regeneration. Moreover, manipulating the fundamental biochemical pathways involved would require cautious assessment, for fear of unintended consequences. Nevertheless, current knowledge provides guiding principles for strategies to optimise liver regeneration potential.

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Core Tip: The liver has remarkable regenerative potential, allowing recovery from 90% hepatectomy in some rodent models. Current understanding of liver regeneration comes from *in vitro* and animal models. Liver regeneration is controlled by mitogens acting in autocrine, paracrine, and endocrine ways. Complex cross talk occurs between parenchymal and non-parenchymal cells. Regeneration involves hypertrophy and hyperplasia, with both cellular phenotypic fidelity and transdifferentiation, which come into play according to the nature and magnitude of the injury, and the presence of underlying liver disease. Current knowledge provides guiding principles for strategies to optimise liver regeneration potential in the treatment of liver tumours.

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INTRODUCTION

The process of liver regeneration is highly complex, and incompletely understood. Moreover, the components of this complexity are multiple. Firstly, liver regeneration may be triggered by a wide range of diverse injury types, occurring in isolation or combination, and including physical trauma, infection, inflammatory processes, direct toxicity, and immunological insults. Commensurate with the range of injuries, the biochemical mechanisms which trigger liver regeneration in the first place are also diverse, but only partly identified. Second, the response to injury is not only dependent on the type of injury, but also its magnitude. For example, liver growth after 30% partial hepatectomy in the rat model is predominantly by hepatocyte hypertrophy (liver growth by hepatocyte volume increase), in contrast to the hyperplasia (liver growth by hepatocyte proliferation) seen after 70% hepatectomy. The mechanism underlying this observation is poorly understood. Third, even in the context of liver regeneration by proliferation, different pathways are activated depending on the magnitude of the injury and the status of the background liver. Thus, when the default pathway of phenotypic fidelity (hepatocytes dividing to produce more hepatocytes, cholangiocytes dividing to produce more cholangiocytes, and so on) fails, alternative pathways are recruited whereby intrahepatic bipotential cells transdifferentiate to hepatocytes or cholangiocytes to meet the deficit. Fourth, the triggers and drivers to liver regeneration are an expanding multitude of cytokines, hormones, and growth factors (collectively referred to as hepatic mitogens), from hepatic and extra-hepatic sources, acting either synchronously or metachronously, each subject to complicated and ill-defined control mechanisms and feedback loops. The mitogen maelstrom is characterized by much redundancy (ablation of particular mitogens is compensated by others) and overlapping 'biochemical promiscuity' (with mitogens impacting on more than one receptor, or intracellular signalling pathway). This degree of overlap and redundancy is understandably a highly valuable evolutionary adaptation to meet the diverse insults the liver is exposed to. Whilst many mitogens have been identified and characterized, the complexity of the interactions make it extremely difficult to assign quantitative relative contributions or importance. Fifth, the complexity of interactions in mitogenic stimuli is further enhanced by the interplay between parenchymal cells (hepatocytes and cholangiocytes) and non-parenchymal cells [Kupffer cells (KC), hepatic stellate cells (HSC), liver sinusoidal endothelial cells (LSEC)], with the latter group, though present in much smaller numbers, playing critical roles. Sixth, the regenerative response is significantly affected not only by the nature of the injury and its magnitude, but also by the health of the underlying liver. Thus, liver regeneration in the face of established steatosis, steatohepatitis, fibrosis, cirrhosis, or biliary outflow obstruction is much altered to that in healthy liver tissue. Seventh, although the processes driving liver

regeneration are only partially understood, those controlling the stop signals, once the liver has grown sufficiently, are even less well defined. Lastly, although many *in vitro* and animal models are available for the study of liver regeneration, all have their limitations, and their results cannot necessarily be extrapolated to the human situation where information is most limited.

This review provides an overview of liver regeneration biology, and the implications of our current understanding for the treatment of liver tumours. We discuss the subject in separate sections listed below. It is emphasized that the presentation of the subject in this way, though designed to orientate the reader, is somewhat artificial in the context of a biological process characterized by multiple synchronous and overlapping events. There is therefore a degree of overlap between sections, with references made to key events in one section subsequently expanded upon in later ones.

Section 1 describes the models of liver regeneration and provides an account of the in vitro, animal, and human models that provide our current knowledge of liver regeneration.

Section 2 describes the very early events post liver injury (provided by the hepatectomy model) and provides an account of the known early triggers to liver regeneration.

Section 3 provides an account of the multiple hepatic mitogens which contribute to initiating and maintaining liver regeneration.

Section 4 describes the contribution of non-parenchymal cells to liver regeneration.

Section 5 describes the 'alternative pathways' of liver regeneration, in which stem cell trans differentiation is recruited as a mechanism to deal with situations when phenotypic fidelity fails.

Section 6 describes the influence of underlying liver disease to liver regeneration.

Section 7 describes current knowledge of the mechanisms underlying ceasing of liver regeneration.

Section 8 considers how our current knowledge of liver regeneration affects therapy for liver tumours currently and in terms of future developments.

SECTION 1: MODELS OF LIVER REGENERATION

Although the clinician's perspective may aim to use understanding of liver regeneration to optimize and develop therapeutic interventions in humans, much of our current knowledge of liver regeneration is based on animal and *in vitro* models. This section describes the historical evolution of liver regeneration research, the current predominant animal models: Rat, mouse, and zebrafish, the modern tissue culture *in vitro* models, and finally human studies of liver regeneration.

Historical evolution of liver regeneration research

Early research and the flow theory: The history and evolution of animal models used for the study of liver regeneration is described in detail within the excellent review by Mortensen *et al*[1]. The very first liver regeneration research is attributed to Nicolas Eck, a 29-year-old Russian military surgeon, in his investigation of portocaval fistula in dogs[2].

From this early period and into the early 1900s, the prevailing view, referred to as 'the flow theory' hypothesized that liver homeostasis and regeneration could be maintained provided that the liver sinusoids were supplied with mechanical flow of blood, irrespective of its source.

The theory was seemingly supported by experiments showing liver regeneration in dogs after 70% hepatectomy who had undergone total portocaval transposition (thus delivering exclusively systemic venous blood to the sinusoids)[3], and by similar experiments showing liver regeneration in dogs after 42% hepatectomy who had undergone portocaval shunt and arterialization of the hepatic portal stump[4] (thus delivering exclusively arterial blood to the sinusoids). With hindsight, the interpretation of these results was incorrect, in that in both cases, liver regeneration was in fact supported by growth factors of portal origin spilling into the systemic circulation.

The humoral theory: The concept that constituents of portal blood were essential to liver homeostasis and regeneration only gradually gained acceptance, despite early evidence form Hahn who described liver failure in dogs undergoing portocaval shunts [5]. In the 1920s, Rous and Larimore reported that unilateral portal ligation produced ipsilateral atrophy with contralateral hypertrophy in a rabbit model[6]. From the 1960s

onwards many more investigators pursued the idea of portal flow as critical in liver regeneration, in experiments including those of Marchioro *et al*[7], who carried out canine split portocaval transposition in which one portal branch is supplied with venous blood from the inferior vena cava and the second portal branch receives portal blood, showing atrophy and hypertrophy of the respective parts of liver parenchyma. Furthermore it was demonstrated that adjusting flow and oxygenation alone did not, in a dog model, compensate for the absence of portal blood[8].

Characterising portal blood constituents: With the recognition of the importance of portal blood came an impetus to define the source and nature of vital portal blood constituents. Thus splanchnic portal flow separation experiments were carried out separating portal flow of distal stomach, duodenum, pancreas and spleen from that of small intestine, with the overall finding that the grafts supplied with small intestinal portal flow atrophied, in contrast to those supplied with portal blood from the upper intestinal tract[9,10].

Thereafter, searches for candidate hepato-trophic factors were carried out by infusing individual growth factors and hormones in portal deprived parenchyma to see if rescue could be achieved. In this way, it was demonstrated that insulin infusion into one portal branch of liver after portocaval shunt could partially rescued atrophy of the liver[11], though insulin was unable to prevent liver atrophy following complete splanchnic evisceration[12].

This portocaval shunt rescue model of experimentation allowed the identification of other factors which promoted liver regeneration including thyroxine (T 3), insulin-like growth factor II, transforming growth factor alpha (TGFα) and hepatocyte growth factor (HGF)[13]. Although portal in origin, the systemic blood dissemination of the factors involved in liver regeneration were shown in canine experiments with auto-transplantation of small liver grafts to the jejunal mesentery, then randomising animals to sham surgery or 70% hepatectomy. In contrast to sham surgery, autografts in hepatectomised animals did not atrophy, indicating a growth stimulus *via* the systemic circulation[14].

Similar results were obtained in parabiosis experiments. Thus, using rats with surgically conjoined systemic circulations, partial hepatectomy in one rat, resulted in liver hypertrophy in the non-hepatectomised rat[15,16].

Thus, the early experiments establishing the underlying principles of liver regeneration were performed using predominantly large animal models. In the more recent era, small animal models have preferentially been used because, as well as providing similar physiology and anatomy to the large animal models, they presented advantages in terms of cost, animal husbandry, rapidity of experimentation, and, in the mouse in particular, greater opportunity for genetic modification as an investigative tool. The sections below follow on to describe the rat and mouse models, with the subsequent evolution to the zebrafish model.

It should be emphasised that drawing conclusions from these different models presents additional complexity per se, in that the observations of one species model may not necessarily be extrapolated to the others. Moreover, even within one species, different liver injury types may present differing characteristics. For example, in the mouse model, epidermal growth factor receptor (EGFR) blockade markedly inhibits liver regeneration after paracetamol injury[17], but only delays it after partial hepatectomy (PH)[18].

Current predominant animal models: rat, mouse, and zebrafish

Rat model: The rat model has gained favour over larger animal models (*i.e.*, dogs, rabbits, baboons and pigs) due to advantages in terms of ethics, costs, and practicalities such as husbandry, handling, and shorter experimental times[19] although their size renders surgery more intricate.

As early as 1931, Higgins & Anderson described a standardized technique for partial hepatectomy in rats, which resulted in liver regeneration[20]. Two decades later, Bucher *et al*[15] reported on parabiotic experiments, whereby rats that underwent partial hepatectomy were joined to partner rats with intact livers by way of an abdominal wall anastomosis. The authors found that mitosis increased both within the operated and the intact livers, thus concluding that liver regeneration is influenced by factors in the systemic circulation. In a contemporaneous report of parabiotic rats, Wenneker & Sussman[16] found that liver weight and number of hepatic cells increased both in hepatectomized and "normal" rats, thus reaching the same conclusion. Moolten & Bucher[21] investigated this further by establishing carotid-to-jugular cross-circulation from partial hepatectomy to normal rats, and demonstrating

that DNA synthesis increased in the normal livers, dependent on the extent of hepatectomy in the parabiotic partner. Since these early experiments, a variety of surgical and hepatotoxic rat models have been developed for the study of regeneration in acute and chronic liver disease.

The rat liver consists of four main lobes: middle (38% liver mass), left lateral (30%), right (22%), and caudate (8%)[19]. In descriptions where the paracaval portion is considered separate from the caudate, this amounts to 2% of liver mass. These lobes, and their subdivisions, are analogous to the human liver segments described by Couinaud[22]. Specifically, the caudate lobe (which consists of the Spiegel lobe and paracaval portion) corresponds to the human segments (Sg) I and IX, the left lobe to Sg II, the left component of the middle lobe to Sg III, the right component to Sg IV, V, and VIII, and the right lobe to Sg VI and VII[23].

The classical surgical model involves a 70% (2/3) hepatectomy, as described by Higgins & Anderson[20], and remains the most common surgical model for liver regeneration. Impressively, rat liver can completely regenerate within 8 d of 70% hepatectomy[24]. Variations to this model can result in 5%-97% partial hepatectomies, depending on the combination of liver lobes resected[19]. Impressively, 90% hepatectomy in rats is survivable[25]. Furthermore, survival can (perhaps counterintuitively) be enhanced by suppressing the abrupt early regenerative response of the remnant liver *via* the mitogenactivated protein kinase pathway, thus rendering regeneration linear in the acute phase[26] or by selective bowel decontamination with gentamicin[27]. These phenomena point towards a substantial regenerative reserve in rats, which unfortunately is not found in humans and which limits extrapolation from rodent models to humans. Bile duct ligation (BDL) is another commonly used surgical model, which involves dividing the common bile duct between ligatures, thus providing a model for the study of cholestatic disease[28]. Yet another surgical model is portal branch ligation, after which ipsilateral atrophy and contralateral hyperplasia is observed in rats[29] analogous to human clinical scenarios such as portal vein embolisation (PVE) or associating liver partition and portal vein ligation for staged hepatectomy (ALPPS).

Hepatotoxic models have been extensively studied in both rats and mice, shown in Table 1, with the aim of replicating acute or chronic liver disease. Their mechanisms are also described below in the context of mouse models. The hepatotoxic approach has been used to demonstrate the protective effects of flavonoids[30], thiamine[31] protocatechuic acid[32], *Lactococcus lactis* in probiotic preparation[33] and 5-methoxytryptophan[34], to mention a few examples. An alternative approach to hepatotoxicity is the manipulation of the cell cycle. Specifically, 2-acetaminofluorene (AAF) has been shown to inhibit hepatocyte proliferation, whilst inducing the proliferation and transdifferentiation of oval cells (hepatic progenitor cells) to hepatocytes after partial hepatectomy[35,36], thus shedding light on alternative liver regenerative pathways.

Mouse model: Although much knowledge on liver regeneration has been generated from partial hepatectomy rat models, the mouse model provides an attractive alternative due to lower costs (mice generally require fewer expensive reagents and less expensive housing)[39] relative ease of handling, and immense experimental potential afforded by genetically altered (transgenic and knockout) mice[19].

Mouse models of liver regeneration have been described in various contexts, including: partial hepatectomy[40], portal branch occlusion[41], bile duct ligation[42], chemical, pharmacological or immune-mediated injury[43-47], and chronic conditions such as non-alcoholic fatty liver disease[48] and liver cancer[49].

The lobar anatomy of the mouse and rat liver is broadly similar, and the inferior vena cava is intrahepatic in both species[39]. A significant distinction is the absence of a gallbladder in the rat[19]. In the mouse, the normal liver consists of seven lobes with the following mass proportions: left posterior (37%), left anterior (12%), right anterior (22%), right posterior (14%), right middle (8%), and two omental lobes (7%)[50]. The classical surgical model in rodents is the partial hepatectomy, which most commonly results in removal of 70% of the liver mass (also referred to as a "2/3 hepatectomy") [19]. Portal branch occlusion can be performed radiologically in humans and in large animals but requires an open surgical approach in rodents[19]. BDL has also been developed as a model of cholestasis[42], with relevance to the study of malignant biliary obstruction. Although the total BDL rat model has existed for decades, mice have been used more recently in the partial BDL model, whereby (rather than transecting the bile duct between ligatures) a 7-0 needle is ligated onto the duct. When the needle is removed, a reproducibly narrow bile duct lumen is left, which results in less liver necrosis[42] and may more closely resemble chronic cholestasis. These surgical models are of particular interest with regards to single or staged hepatec-

Table 1 Hepatotoxins used in rodent models

Toxin	Mechanism	Necrosis pattern
Acetaminophen (paracetamol)[19,36,37]	Free radical enhancement and Kupffer cell activation	Pericentral
Carbon tetrachloride[19,30,37]	Free radical enhancement and Kupffer cell activation	Pericentral
Concanavalin A[37]	T-cell activation; cytokine release; ICAM-1 & VCAM-1 upregulation.	Centrilobular
D-Galactosamine[19,37]	Uridine metabolite deficiency	Random
Ethanol[19,31]	Increases production of reactive oxygen species and infiltration of inflammatory cells	None
Lipopolysaccharide[37]	Kupffer cell activation	Centrilobular
Thioacetamide[19,37,38]	Increases production of toxic metabolites and reactive oxygen species	Pericentral

ICAM-1: Intercellular adhesion molecule 1; VCAM-1: Vascular cell adhesion molecule 1.

tomies with or without portal vein occlusion in human patients with liver tumours, where physiological reserve, oncological and technical resectability, as well as liver tumour burden and status of background parenchyma will determine the most optimal approach. However, recapitulating human liver procedures in mouse models is limited by the fact that mice are relatively very small, and (as in humans) there is significant anatomical variability in their hepatic vascular and biliary systems[39]. Furthermore, rodents can typically survive with much smaller liver remnants than humans, and the kinetics of liver regeneration vary between species. Nevertheless, surgical techniques in mice are well established and are characterized by reproducibility and minimal operative mortality[39].

The most frequently used hepatotoxins used to induce liver injury in mouse models are carbon tetrachloride (CCl_4), d-galactosamine, paracetamol (acetaminophen), ethanol[51] and thioacetamide[19]. CCl_4 can induce acute and chronic liver injury through its action on cytochrome P450, leading to the production of free peroxide radicals which cause lipid peroxidation of hepatocyte[19]. The disadvantage of the CCl_4 model is the inflammatory and immune response caused during hepatocyte injury, which may confound models of liver regeneration[19]. D-galactosamine is thought to induce liver injury *via* intracellular deficiency in uridine metabolites and can additionally induce hepatocyte apoptosis when combined with lipopolysaccharide [19]. Paracetamol is metabolized by cytochrome P450 and in overdose leads to toxic levels of N-acetyl-benzoquinone imine, free radical formation and centrilobular apoptosis/necrosis[19]. The kinetics of liver regeneration after CCl_4, D-Galactosamine and paracetamol-induced injury are similar[19]. Ethanol induces liver injury *via* mitochondrial dysfunction, oxidative stress, inflammatory cell infiltration and translocation of intestinal bacteria which can then enter the portal and systemic circulation [19]. Finally, thioacetamide leads to oxidative stress *via* its conversion to thioacetamide disulfoxide which increases the production of reactive oxygen species[19].

In addition to the hepatotoxic models mentioned above, several dietary models are used in mice to model liver disease. These include the 1,4dihydro2,4,6trimethylpyridine-3,5-dicarboxylate (DDC) diet, which leads to biliary injury and regeneration [52], the modified choline-deficient ethionine diet, which leads to hepatocellular injury, steatosis and spread of ductular cells from the portal tract[53]. More recently, a mouse model with rapid progression from normal liver to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) within 24 wk has been described by Tsuchida *et al*[49]. This was achieved by feeding C57BL/6J mice a western diet (high-fat, high-fructose and high- cholesterol) and administering weekly intraperitoneal doses of CCl_4.

The development of transgenic and knockout mouse models has enabled closer scrutiny of pathophysiological mechanisms with regards to liver regeneration after surgery or chemical/diet-induced injury, also highlighting the importance of the innate and adaptive immune system in liver regeneration[54].

The opportunities offered by these models and their relevance to the treatment of liver tumours in humans will be elaborated in the sections to follow. Table 2, whilst non-exhaustive, gives an impression of the breadth and potential of transgenic and KO mouse models in the study of liver regeneration.

Zebrafish model: Following their discovery in the Ganges River in the late 19th century, zebrafish (Danio rerio) were initially used by embryologists to investigate

Table 2 Studies of liver regeneration involving transgenic or knockout mice

Yr	First author	Gene product	Study title	Ref.
1994	Webber	TGF-α	"Overexpression of transforming growth factor-alpha causes liver enlargement and increased hepatocyte proliferation in transgenic mice"	[55]
1996	Cressman	IL-6	"Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice"	[56]
1997	Yamada	TNF	"Initiation of liver growth by tumor necrosis factor: deficient liver regeneration in mice lacking type I tumor necrosis factor receptor"	[57]
1998	Greenbaum	C/EBP-β	"CCAAT enhancer-binding protein beta is required for normal hepatocyte proliferation in mice after partial hepatectomy	[58]
1998	Rai	iNOS	"Impaired liver regeneration in inducible nitric oxide synthase-deficient mice"	[59]
1998	Roselli	uPA	"Liver regeneration is transiently impaired in urokinase-deficient mice"	[60]
1998	Yamada	TNFR-1TNFR-2	"Analysis of liver regeneration in mice lacking type 1 or type 2 tumor necrosis factor receptor: requirement for type 1 but not type 2 receptor"	[61]
2002	Anderson	PPAR-α	"Delayed liver regeneration in peroxisome proliferator-activated receptor-alpha-null mice"	[62]
2003	Leu	IGFBP-1	"Impaired hepatocyte DNA synthetic response posthepatectomy in insulin-like growth factor binding protein 1-deficient mice with defects in C/EBP beta and mitogen-activated protein kinase/extracellular signal-regulated kinase regulation"	[63]
2003	Strey	C3a/C5a	"The proinflammatory mediators C3a and C5a are essential for liver regeneration"	[64]
2004	Borowiak	Met	"Met provides essential signals for liver regeneration"	[65]
2004	Mohammed	TIMP3	"Abnormal TNF activity in Timp3(−/−) mice leads to chronic hepatic inflammation and failure of liver regeneration	[66]
2004	Nakamura	OSM	"Hepatocyte proliferation and tissue remodeling is impaired after liver injury in oncostatin M receptor knockout mice"	[67]
2004	Oe	TGF-β	"Intact signaling by transforming growth factor beta is not required for termination of liver regeneration in mice"	[68]
2005	Duffield	DTR	"Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair"	[69]
2005	Mitchell	HB-EGF	"Heparin-binding epidermal growth factor-like growth factor links hepatocyte priming with cell cycle progression during liver regeneration"	[70]
2005	Oliver	MT	"Impaired hepatic regeneration in metallothionein-I/II knockout mice"	[71]
2005	Seki	MyD88	"Contribution of Toll-like receptor/myeloid differentiation factor 88 signaling to murine liver regeneration"	[72]
2006	Fernández	Caveolin-1	"Caveolin-1 is essential for liver regeneration"	[73]
2006	Olle	MMP9	"Matrix metalloproteinase-9 is an important factor in hepatic regeneration after partial hepatectomy in mice"	[74]
2007	Mayoral	Caveolin-1	"Dispensability and dynamics of caveolin-1 during liver regeneration and in isolated hepatic Cells"	[75]
2009	Tumanov	Rag1LT	"T cell-derived lymphotoxin regulates liver regeneration"	[54]
2010	Erhardt	CCR5, CXCR3	"Tolerance induction in response to liver inflammation"	[47]
2010	Liu	GPC3	"Suppression of liver regeneration and hepatocyte proliferation in hepatocyte-targeted glypican 3 transgenic mice"	[76]
2012	Borude	FXR	"Hepatocyte-Specific Deletion of Farnesoid X Receptor Delays But Does Not Inhibit Liver Regeneration After Partial Hepatectomy in Mice"	[77]
2013	Bhave	GPC3	"Regulation of Liver Growth by Glypican 3, CD81, Hedgehog, and Hhex"	[78]
2014	Kong	FGF15	"Fibroblast growth factor 15 deficiency impairs liver regeneration in mice"	[79]
2014	Yang	Lrp5/6	"β-catenin signaling in murine liver zonation and regeneration: a Wnt-Wnt situation!"	[80]
2015	Lu	Mdm2	"Hepatic progenitor cells of biliary origin with liver repopulation capacity"	[81]
2016	Swiderska-Syn	Cre recombinase	"Hedgehog regulates yes-associated protein 1 in regenerating mouse liver"	[82]
2018	Tsagianni	MET	"Combined Systemic Disruption of MET and Epidermal Growth Factor Receptor Signaling Causes Liver Failure in Normal Mice"	[83]
2019	Asrud	Epac	"Mice depleted for Exchange Proteins Directly Activated by cAMP (Epac) exhibit irregular liver regeneration	[84]

			in response to partial hepatectomy"	
2019	Fortier	p38α MAPK	"Hepatospecific ablation of p38α MAPK governs liver regeneration through modulation of inflammatory response to CCl 4-induced acute injury"	[85]
2019	Modares	IL-6R	"IL-6 Trans-signaling Controls Liver Regeneration After Partial Hepatectomy"	[86]
2019	Zhou	Rictor	"Mammalian Target of Rapamycin Complex 2 Signaling Is Required for Liver Regeneration in a Cholestatic Liver Injury Murine Model"	[87]
2020	Laschinger	CGRP-RAMP1	"The CGRP receptor component RAMP1 links sensory innervation with YAP activity in the regenerating liver"	[88]
2020	Seguin	Mfrn1, Mfrn2	"The mitochondrial metal transporters mitoferrin1 and mitoferrin2 are required for liver regeneration and cell proliferation in mice"	[89]
2020	Xue	GPC3	"Phosphorylated Ezrin (Thr567) Regulates Hippo Pathway and Yes-Associated Protein (Yap) in Liver"	[90]

developmental biology[91]. Their relative low cost, rapid development from one-cell embryo to free-swimming larva 5 d post-fertilisation, optical transparency enabling direct observation using light and fluorescent microscopy, and relative genetic conservation compared to the human genome with approximately 70% of human genes having a zebrafish orthologue[92] has led to their role within medical research expanding considerably. In the realm of liver biology, applications include the study of high throughput drug discovery and hepatotoxicity screening, forward genetic screening, heritable and developmental liver diseases, the molecular and cellular factors that contribute to human liver disease, liver cancer biology and liver regeneration[91,93-95]. The research opportunities and disadvantages presented by zebrafish are summarized in Table 3.

Cell types are highly conserved between zebrafish and mammalian livers, with the exception of hepatic immune cells (Kupffer cells), which have not been identified in zebrafish. Whilst zebrafish provide immensely useful models, this difference highlights the caution needed in the extrapolation of results between species. As discussed below, Kupffer cells play an important part in cytokine priming of hepatocytes, implying that a different priming mechanism operates in zebrafish, or that this role is played by different cell type. Cellular morphology and physiology are also largely conserved with zebrafish livers demonstrating similar functions to mammalian livers including secretion of bile, glycogen and lipid storage, insulin responsiveness, ammonia metabolism and the production and secretion of proteins including complement, clotting factors and a protein resembling albumin. The morphological composition of the zebrafish, however, is distinct to the mammalian liver with the liver arranged into 3 Lobes that lack a pedicle that separates the lobes in the mammalian liver. Moreover, the portal architecture of mammalian livers is not observed. In fish, the hepatocytes are arranged into tubules with bile ductules running between two rows of hepatocytes[91].

Liver regeneration in mammalian livers involves a compensatory regeneration with hepatocyte proliferation and hypertrophy. In contrast, zebrafish demonstrate true epimorphic regeneration in response to partial hepatectomy with regrowth of the resected lobe, again highlighting significant inter-species differences. Genome-wide gene expression studies have demonstrated that liver regeneration is the result of a coordinated expression of thousands of genes, and whilst several pathways have been identified as important in liver regeneration in both mammals and zebrafish including WNT, fibroblast growth factor receptor (FGFR) and bone morphogenic protein receptor, in isolation they are insufficient to drive the complex process of liver regeneration. The mechanisms underlying the difference between mammalian liver regeneration and zebrafish, and epimorphic regeneration are still to be elucidated[96].

In vitro models

The observation of interspecies variability, the ethical guiding principles of the 3R principles (replacement, refinement, and reduction of animal testing), and the opportunity of better-defined experimental conditions have motivated the development *in vitro* models to study liver biology. Thus, two- and three-dimensional (2D and 3D) *in vitro* models have increased our understanding of the mechanisms of liver injury, hepatotoxicity, and mechanisms of liver regeneration[97].

2D culture models: 2D *in vitro* liver models, have traditionally used immortalised cell lines such as the HepG2 and the HepaRG cell lines derived from human progenitor cells[98], or mechanically and enzymatically dissociated primary cells[99] expanded on

Table 3 Advantages and disadvantages of zebrafish as a model for human liver pathophysiology

Advantages	Disadvantages
Vertebrate body plan	Partial genome duplication in teleosts
Ease of husbandry	Differences in microanatomy and liver architecture
Inexpensive to maintain	Less conserved physiology than mammalian models
Large numbers of embryos produced rapidly	Less conserved morphogenesis than mammals
External development	Less developed cell culture technology
Optical clarity during development	Poorly developed embryonic stem cell technology
Zebrafish liver not required for foetal haematopoiesis	
Amenable to forward and reverse genetics	
Molecular conservation of development	
Amenable to high-throughput screening: (1) Phenotype assessment; and (2) Drug/chemical screening	

plastic surfaces, or supported by extracellular matrix (ECM) scaffolding[100]. Though presenting advantages in terms of ease of tissue culture, such systems have limitations; for example, cells lines have fundamentally different gene expression profiles to primary hepatocytes, owing to their immortalised nature[101]. Primary hepatocytes have some benefits in this regard, but are difficult to source (in the human case), exhibit donor variability[102], and rapidly lose their differentiation and function (such as morphology and toxicant related genes expression) in plastic culture[100,103,104].

The presence of ECM partially addresses these shortcomings. Culturing primary hepatocytes between two layers of collagen, termed sandwich-cultured hepatocytes (SCH)[105], results in extended viability, retained cellular polarity with correct localization of basolateral and canalicular transporters[106] as well as formation of functional bile networks[107]. However, despite their promising properties compared to monolayer cultures, sandwich cultures have their own disadvantages including the barrier to introduced materials created by the collagen layers, and low levels of expression of cell-to-cell adhesion proteins that are critical for cell function and differentiation[108]. As such the role of sandwich culture in the experimental process is often limited to short term studies.

3D culture models: Significant progress has been made using 3D *in vitro* hepatic models with benefits in terms of maturity of hepatocytes, long term viability, and more precise representations of the microenvironment of the *in vivo* liver[109]. *In vitro* liver modelling studies with human cells have allowed investigation of liver development, liver disease modelling, liver regeneration, and therapeutic transplantation. Given the complex 3D structure and functional regionalization of the liver, 3D liver models including organoids offer the advantage of more closely recapitulating spatial organization, important cell-cell and cell-ECM contacts that stimulate proliferation, differentiation, liver specific expression, and responsiveness to exogenous stimuli[110]. These advantages have been further emphasised by the use of coculture in such systems, allowing the inclusion of key non-parenchymal cells[97].

Human-induced pluripotent stem cells (hIPSCs) offer an effectively unlimited source of genetically diverse cell lines that can be generated from both healthy and diseased livers. Furthermore, these cells are amenable to genetic modification using the CRISPR technology in order to facilitate disease modelling[111]. hIPSCs have further expanded the opportunity for 3D *in vitro* culture systems by the development of hepatic organoids from hIPSCs[112]. Thus, reports describe the design of organoids involving multiple cell types by co-differentiating hepatocytes and cholangiocytes [113], or hepatocytes with other supporting cell types including stellate-like and Kupffer-like cells[114]. However, an important limitation is the relative immaturity of hepatocyte-like cells generated from hIPSCs. This is demonstrated by continued alpha-fetoprotein (αFP), low albumin expression, and distinctive CYP expression and function[115]. The problem of functional maturity has been partially addressed by modifying culture conditions, including the medium composition (*e.g.*, inclusion of specific growth factors, hormones)[115]. More recent approaches to circumvent the disadvantages of hIPSCs have involved the use of primary cells to form organoids.

Thus, Huch *et al*[116] generated human liver organoids from primary ductal Epidermal Cell Adhesion Molecule positive cells grown in a defined human liver media allowing culture with stable function for over 6 mo, and Justin *et al*[117] describe the formation of biliary organoids from primary cholangiocytes.

In spite of these advances, 3D *ex vivo* cultures do carry their own drawback including difficulties in controlling cellular distribution, innervation, and vascularization-with the latter aspect of particular importance given the central role of the liver sinusoids to hepatic function.

Repopulation of decellularised livers: Repopulation of decellularised liver scaffolds with cells has offered a further refinement to the *in vitro* investigation of liver regeneration as well as potential therapeutic opportunities[118].

Earlier approaches to generating functional livers include hepatocyte transplantation in humans[119], as well as in animals[120]. In the latter case, microcarriers and biodegradable polymer scaffolds have been described, resulting in albumin production and clearance of bilirubin and urea metabolites[120]. These efforts have laid the ground for three-dimensional scaffolds[121] which are either biological membranes[122], collagen sponges[123], or synthetic hydrogels[124], and which enable the production of hepatic organoids. In another approach to liver regeneration, chimeric murine models have been developed, whereby mouse liver is extensively repopulated with human hepatocytes, thus permitting the study of liver disease (*e.g.*, viral hepatitis) within humanised organs *in vivo*[125].

More recently, techniques in whole liver decellularization and repopulation have moved the field further, although significant challenges remain. In general terms, the process involves removal of the liver's cellular and immunogenic components, thus creating a scaffold which retains the ultrastructure and properties of the ECM[126]. This is usually followed by static cultivation of cells (*e.g.*, hepatocytes) and their subsequent infusion into the scaffold.

In one of the first such studies, Uygun *et al*[127] demonstrated that ischaemic rat livers can be decellularised whilst preserving structural and basement-membrane-based components of the ECM, as well as the microvasculature. The investigators achieved decellularisation by portal vein perfusion using sodium dodecylsulfate (an anionic detergent), and repopulation with primary rat hepatocytes *via* the same route. Recellularised grafts were implanted in rats for 8 h, and after explantation underwent *ex vivo* blood perfusion for 24 h, demonstrating ongoing hepatocyte metabolic activity. Others have demonstrated that implanting repopulated ECM liver scaffolds into rats which had undergone extended hepatectomy improved liver function and extended their mean lifespan from 16 to 72 h[128]. In the last 10 years, a variety of animal models, decellularisation techniques, repopulation routes and cell sources have been described, with promising outcomes in terms of vascular repopulation[118,129,130], hepatocyte survival[131] as well as formation of biliary duct-like structures and activation of liver detoxification enzymes[132]. One of the commonest sources of liver scaffolds is the rat[118,127,132-137] repopulated with rat hepatocytes (although cholangiocytes[136] and lineages from pluripotent stem cells, mesenchymal cells, and fibroblasts have also been described[137] usually *via* the portal vein. With regards to human tissue, Verstegen *et al*[138] demonstrated that decellularised human livers can be repopulated with human umbilical vein endothelial cells, leading to re-endothelialisation of the vascular tree. Table 4 presents further examples of the different approaches to liver decellularisation-repopulation developed thus far.

The main challenges in producing a viable whole organ from liver decellularisation-repopulation techniques include heterogeneity of cell engraftment, thrombosis (partly related to incomplete or suboptimally functional endothelium as well as microvascular injury[121,130]), the re-creation of an intact and functional biliary tree, as well as attaining the specific distribution of liver cell types seen in the native healthy organ. Mesenchymal and pluripotent stem cells for repopulation are currently considered attractive research avenues[121] as they may lead to more clinically applicable models.

Human models

The study of human liver regeneration is limited to observational data in the context of clinical pathology and applied therapies, and thus contrasts to the directed experimental approaches possible in animal models. Moreover, access to human liver tissue during the regenerative process is not possible as liver biopsy can only be justified by clinical need, given the risks of the procedure including a measurable mortality[139]. The available observational data comes from a combination of clinical findings, serum biomarker measurements, and imaging. Clinical observations and blood bio markers are subject to difficult interpretation because of the confounding effects of the hetero-

Table 4 Examples of liver decellularization-repopulation studies

First author	Yr	Liver scaffold source	Cell source & type	Repopulation route	Outcomes	Ref.
Uygun	2010	Rat	Rat hepatocytes	Portal vein	Recellularised liver grafts implanted in rats, perfused in vivo for 8 h, explanted and assessed after 24 h, demonstrating hepatocyte survival, albumin secretion, urea synthesis and cytochrome P450 expression.	Uygun 2010 [127]
Zhou	2011	Mouse	Human foetal hepatocytes	Portal vein	Recellularised liver matrix implanted in mice, achieving hepatocyte survival after 6 wk, with albumin secretion and cytochrome P450 expression.	Zhou 2011 [131]
Ko	2014	Pig	Murine endothelial cells, after scaffold conjugation with rat anti-mouse CD31 antibodies	Portal veinHepatic arteryInferior vena cava	Recellularised liver grafts implanted in pigs, demonstrating good blood flow and patency throughout vascular network over 24 h after transplantation.	Ko 2015 [130]
Navarro-Tableros	2015	Rat	Human liver stem-like cells	Portal vein	Loss of embryonic markers, expression of albumin, lactate dehydrogenase and cytochrome P450 subtypes. Production of urea and nitrogen.	Navarro-Tableros 2015[133]
Ogiso	2016	Rat	Mouse hepatocytes	Biliary tree; Portal vein	(1) > 80% of cells seeded *via* biliary tree entered the parenchyma; (2) Approximate 20% of cells seeded *via* portal vein entered the parenchyma; and (3) Increased gene expression of foetal hepatocyte albumin, glucose 6-phosphatase, transferrin, cytokeratin 19, and gamma-glutamyl transpeptidase, activation of liver detoxification enzymes, formation of biliary duct-like structures.	Ogiso 2016 [132] [PMID 27767181]
Verstegen	2017	Human	Human umbilical vein endothelial cells.	-	Re-endothelialisation of vascular tree, demonstrated by luminal vimentin and von Willebrand Factor/F8 staining.	Verstegen 2017[138]
Butter	2018	Rat	Rat hepatocytes	Hepatic artery and portal vein	In vitro demonstration of hepatocyte spread to all liver lobes, with proliferation, and production of aminotransferases, lactate dehydrogenase and albumin.	Butter 2018 [134]
Chen	2018	Rat	Rat hepatocytes	Portal vein	None (description of materials and methods).	Chen 2018 [135]
Chen	2019	Rat	Rat cholangiocytes Rat hepatocytes	Common bile duct; Portal vein	In vitro viability and function demonstrated by albumin and urea secretion, and gene expression of functional proteins.	Chen 2019 [136]
Harper	2020	Rat	Rat bone marrow cells	Portal vein	Stem cells engrafted in portal, sinusoidal and hepatic vein compartments, achieving expression of endothelial cell surface markers for up to 30 d.	Harper 2020 [118]
Takeishi	2020	Rat	Human hepatocytes, biliary epithelial cells, and vascular endothelial cells derived from pluripotent stem cells, mesenchymal cells, and fibroblasts.	Biliary tree; Portal vein; Central veins	Auxiliary grafts implanted in rats, achieving in vivo functionality for 4 d.	Takeishi 2020[137]

geneity of the study population, diverse pathologies, and varied clinical scenarios even within a defined patient group. Although there are reports of serum biomarkers such as αFP and micro RNAs correlating with liver regeneration, their clinical applicability remains to be established. Combining clinical and serological measurements, scores such as the Acute Liver Failure Study Group index has allowed the identification of patients likely to require liver transplant[140-142].

In this context, the relatively non-invasive nature of modern imaging techniques has provided the main means of assessing liver growth and function, as markers of regeneration. Although liver function correlates well with liver volume in uncompromised livers, this relationship is less clear in patients with pre-existing parenchymal liver[143,144]. Estimation of remnant liver function instead of remnant liver volume is a better predictor of clinical outcome after liver resection in patients with decreased liver function[145]. In order to avoid PHLF, clinicians must ensure that the future remnant liver (FRL) will be sufficient to sustain life. Traditionally, this functionality assessment is made by pre-operatively measuring the volume of the FRL as a surrogate measure of functionality[144]. Volumetry, however, assumes liver parenchymal homogeneity and normal underlying liver function, which are not always present in patients undergoing extensive hepatic resections. This lack of

homogeneity in hepatic function can cause a discrepancy between FRL volume (FRL-V%) and FRL function (FRL-F)[146] which is especially important in patients who present with pre-existing liver disease or who have previously received chemotherapy that resulted in steatotic or microvascular liver changes[146]. As such, FRL-V% cut-off values may not accurately predict the quality of the FRL in some patients, with implication on the development of PHLF and associated mortality. The radiological modalities most used to predict the FLR are outlined below.

Standard liver volumes can be calculated from the patient's body surface area or mass using the formulas originally proposed by Vauthey *et al*[144]. However, these formulas are limited by subject demographics (healthy individuals) and by their modest correlation to liver sizes calculated by more advanced forms of volumetry [147]. CT volumetry of the liver was first performed on cadavers by Heymsfield *et al* [148] in 1979 and was shown to be accurate within 5% of water displacement volumetry. CT is more commonly used due to its greater accessibility, higher spatial resolution, and short acquisition time. MRI, conversely, offers multiple contrast mechanisms and the ability to assess vascular and biliary anatomy in addition to parenchymal pathology. Additionally, MRI also minimises the risk of contrast induced nephrotoxicity and eliminates concerns of radiation exposure[149]. Liver segmentation has emerged as the preferred technique CT volumetry can be used to calculate the volume of the FLR and is widely used to exclude patients from liver resection or to select patients who will benefit from a procedure to increase the volume of the future remnant, such as PVE[150]. However, the outcomes of previous reports correlating the findings of CT volumetric analysis of the future remnant with post-resectional outcome, have not been consistent and the role alternative imaging modalities has been examined[150,151].

Hepatobiliary scintigraphy using 99mTc-iminodiacetic acid analogues, such as 99mTc-mebrofenin, can be used to measure segmental liver function. 99mTc-mebrofenin is excreted into the bile by adenosine triphosphate–dependent export pumps the multidrug-resistance-associated proteins 1 and 2 without undergoing biotransformation during transit through the hepatocytes[146,152,153] . Previous reports in the literature have shown that 99mTc-mebrofenin hepatobiliary scintigraphy (HBS) can provide clinicians with information on FRL-F instead of volumetric information alone[153]. HBS provides visual and quantitative information of global and regional liver function as well as excretory function (intrahepatic and extrahepatic bile transport). 99mTc-mebrofenin is intravenously injected and consequently excreted in bile by the hepatocytes without undergoing biotransformation. As such, the clearance measurement of Technetium-99m mebrofenin using scintigraphy can quantify hepatic function[146,153]. FRL-F assessment using HBS has been proven to be superior to volumetry in the prediction of PHLF and PHLF(M), making HBS the imaging modality of choice prior to proceeding with major hepatectomy. Reports in the literature have illustrated that an HBS cut-off value of 2.7%/min/m^2 can outperform volumetry cut-off values in the prediction and prevention of PHLF and PHLF(M) by identifying high-risk patients with borderline predicted remnant liver function, and consequent selection for pre-operative PVE or other hypertrophic strategies (*e.g.*, ALPPS[154,155]. Certain hepatobiliary units have already implemented HBS in favor of CT volumetry before hepatic resection based on emerging evidence in the literature.

SECTION 2: EARLY EVENTS POST LIVER INJURY AND TRIGGERS TO LIVER REGENERATION

The PH model in rodents has allowed the examination of immediate events which occur within minutes of liver resection and provides an insight into the mechanisms that trigger the process of liver regeneration. These early events relate to vascular portal flow, tissue hypoxia, haemostatic mechanisms, and changes in extracellular matrix integrity.

Vascular events

Following PH, the increased portal blood flow through the remnant liver exerts a heightened shear stress on the LSECs[156]. Shear stress on LSEC induces numerous physiological changes[157] including microscopically visible ones such as increased sinusoidal diameter and changes to LSEC fenestrae and sieve plates[158,159]. Shear stress also induces biochemical responses including the release of vascular endothelial cell growth factor (VEGF) from LSEC[160], the secretion of VEGF and HGF from

hepatic stellate cells[161], and the LSEC production of nitric oxide (NO) by Nitric Oxide synthase (NOS), which increases hepatocyte sensitivity to HGF[162,163]. The physiological importance of NO is suggested by the finding that inhibition of NOS severely impairs liver regeneration in mice after PH[59].

Shear stress also induces the hepatocyte priming cytokine interleukin 6 (IL6) in LSEC[164], as well as expression in of liver regeneration associated WNT, VEGF, and epithelial cell adhesion molecules in hepatic progenitor cells[165].

Another consequence of increased portal flow through the remnant liver is increased exposure to lipopolysaccharide (LPS), which is derived from gut bacteria, and which translocates from the gut into portal blood. PH increases the concentration of LPS in the remnant liver not only because of diversion of more portal blood to the remnant liver, but also because the rise in portal pressure increases intestinal permeability, allowing greater LPS translocation[166,167]. In the sinusoids, LPS binds Toll like receptors (TLR) on Kupffer cells, resulting in the secretion of the hepatocyte priming cytokines IL6 and tumour necrosis factor alpha TNFα[168], in a signalling pathway that is dependent on myeloid differentiation factor 88[169].

The increased expression of liver regeneration promoting biochemicals is not confined to the liver. Following PH in the rat, increased expression of VEGF, HGF, and hypoxia inducible factor (HIF) is also observed portal vein drained tissues such as the spleen and small intestine, whereupon portal VEGF concentrations exceed those of the systemic circulation. The mechanism stimulating this extrahepatic expression of growth factors from portal drained tissues is unclear but may also be related to portal pressure changes[170].

Hypoxia

Following PH, increased portal flow brings about a reflex arterial vasoconstriction (the arterial buffer response), which can result in hypoxia in the remnant liver, given the low partial pressure of oxygen (pO2) in portal venous blood[171].

An important outcome of hypoxia is the induction of HIF, which in turn leads to the activation of multiple genes involved in tissue adaptations to hypoxia ranging from glycolytic metabolism to angiogenesis[172].

In the liver, PH leads to increased expression of HIF and subsequently VEGF[173]. In elegant experiments, Dirscherl *et al*[174] show that the hypoxic environment triggers hepatic stellate cell expression of HIF, resulting in increased expression of VEGF, which then elicits a range of responses in LSEC including proliferation and angiogenesis, but also genes associated with matrix remodelling (discussed in a later section), and LSEC expression of the potent hepatocyte mitogen HGF[174], as well as other liver regenerative genes[175]. Thus, the authors suggest that HSC function as hypoxia sensors in the liver, and trigger angiogenesis in liver regeneration, highlighting the complexity of intercellular cross-talk in this process. In addition, hypoxia induced secretion of complex regeneration promoting molecules from stem cells at extra-hepatic locations may also contribute to promotion of liver regeneration [176].

Haemostasis related factors

The injury to liver tissue in PH results in the activation of mechanisms for haemostasis carried out by platelets and the coagulation cascade. The role of platelets is not confined to haemostasis, but also includes functions relating to liver regeneration [177], and studies in animals and humans suggest impaired liver regeneration in individuals with low platelet counts[178,179].

Following PH, platelets migrate to the space of Disse, where they release liver regeneration promoting biochemical including serotonin, VEGF, and HGF from secreted cytoplasmic granules[180]. In addition to growth factor containing vesicles, platelets contain cytoplasmic RNA, which can be transferred to nearby hepatocytes, resulting in gene expression, and promoting hepatocyte proliferation[181]. Finally, platelets may stimulate liver regeneration by activation of immune cells which also have an important role in cellular cross-talk[180].

In addition to the role of platelets, the coagulation cascade both individually and in combination with 'damage associated molecular patterns' (DAMPs) (including mitochondrial DNA and peptides)[182], activates elements of the complement cascade [183]. These include C3a and C5a, which have a role in stimulating pathways involved in the priming of hepatocytes[184], enabling them to respond to growth factors, as discussed in "*Priming of hepatocytes*".

Other elements of the coagulation cascade may also play key roles in liver regeneration. Thus, Groeneveld *et al*[185] report that intrahepatic deposition of fibrinogen after PH is a key driver to platelet accumulation in the liver. Fibrinogen

depletion was associated with impaired liver regeneration in a mouse model, and in humans undergoing liver resection, low intrahepatic fibrinogen and low post op serum fibrinogen levels were associated with poor liver function an increased mortality.

ECM changes

Urokinase-type plasminogen activator (uPA) activity increases within one minute of PH in rats[186], resulting in the activation of plasminogen to plasmin, which then activates key metalloproteinases (MMP) such as MMP-9[187,188], which remodel the hepatic ECM, where HGF is present in its inactive form. uPA also activates HGF to its active form[189], releasing it locally in the liver parenchyma and also into the circulation in significant quantities[190]. uPA knockout mice show impaired liver regeneration[60]. As well as HGF, the ECM contains other inactive forms of growth factors including HB-EGF and fibroblast growth factor (FGF)[191]. Moreover, the importance of matrix alteration in the initiation goes beyond the release of growth factor stores in the ECM in that hepatocyte response to key growth factors is ineffective in the presence of intact ECM, and that ECM changes are required for growth factor driven hepatocyte proliferation[192,193].

Thus, the rapid action of uPA following PH provides a mechanism to kick start the liver regenerative process by liberating ECM stored growth factors, until such time as other mechanisms begin to contribute to maintaining the liver regenerative process.

SECTION 3: HEPATIC MITOGENS

Liver regeneration is characterized by hypertrophy and rapid proliferation allowing return to the starting volume of liver even if recovering from a 25% remnant in humans, or a 10% remnant in some rodent models. The proliferation of hepatocytes is controlled by a maelstrom of growth factors with different but overlapping effects. Within this complexity exists a hierarchy of functions, whereby hepatocytes first require to be primed ("*Priming of hepatocytes*"), after which they become responsive to a range of mitogens referred to as complete ("*Complete mitogens*") auxiliary ("*Auxiliary mitogens*"), and complex ("*Complex mitogens*"). "*Intracellular signalling pathways*" summarizes the intracellular pathways which transmit the effect of the growth factors described in sections "*Priming of hepatocytes*" - "*Complex mitogens*".

Priming of hepatocytes

Hepatocyte transition from G0 to G1: Although proliferation of hepatocytes is stimulated by a wide range of biochemicals in response to injury, most hepatocytes in uninjured liver do not proliferate[194], although there is some heterogeneity in this regard as discussed in "*Hepatocyte response heterogeneity after PH*" entitled 'hepatocyte regenerative heterogeneity'. The stimulus to proliferation from the multitude of mitogens requires hepatocytes to be 'primed', a complex phenomenon characterized by the induction of > 100 genes[195], which then enables the hepatocytes to respond to these mitogenic stimuli.

Although cell cycle biology is outside the scope of this review, a brief summary of key events is useful to frame the subsequent sections relating to the priming effects of cytokines and proliferative stimulus of mitogens on hepatocytes.

The cell cycle is divided into 2 main phases: mitosis (the actual process of cell division) and interphase (the phase preparing the cell for mitosis). Interphase is further divided into 3 stages, which, in order, consist of the G1 phase (during which the cell synthesises protein and organelles), S phase (during which DNA is replicated) and G2 phase (during which the machinery for mitosis is assembled). Although some cells undergo this cycle continuously, others exit the cycle and enter a stationary phase G0. In order for a cell in G0 to replicate, it first needs to be 'primed' by molecular signals to return to G1, whereupon a different set of signals will determine the speed of replication and how long it continues.

Hepatocytes provide an example of this situation, and are, in the absence of injury, almost entirely in G0[194]. Their proliferation therefore requires priming factors to return them to G1. The priming function is carried out by cytokines TNFα and IL6.

Thus the current working model[184] (illustrated in Figure 1) suggests that the cytokine priming mechanism starts with the activation of nuclear factor-kappa B (NFκB) in Kupffer cells. NFκB activation may be triggered by several stimuli including (1) Binding of TNFα to its receptor; (2) Binding of complement components C3a & C5a to their receptor; or (3) Binding of lipo-polysaccharide to the TLR receptor. Activation

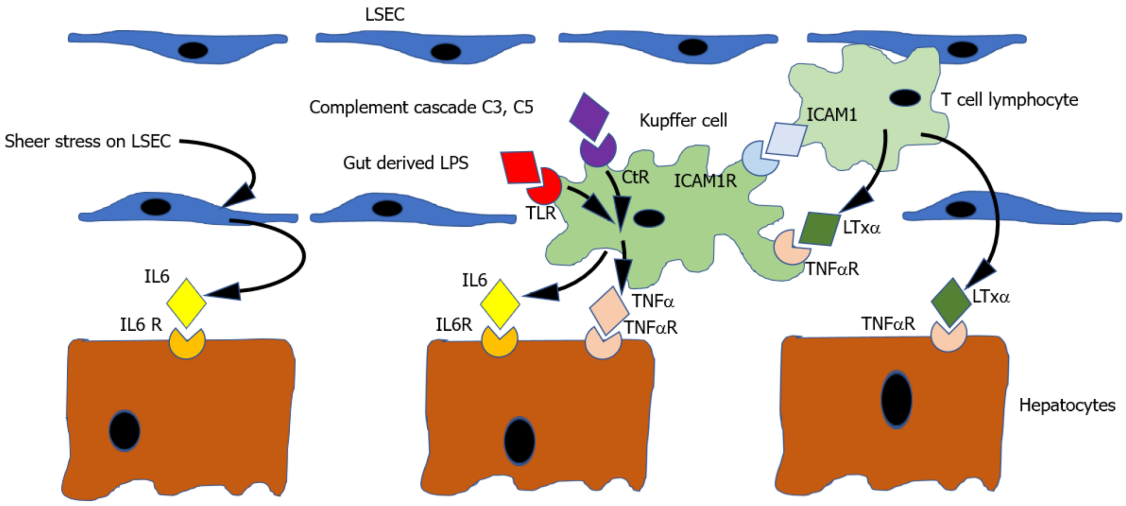

Figure 1 Cytokine priming of hepatocytes. PH induced increase in portal pressure exerts sheer stress on LSEC inducing IL6 secretion. Gut derived LPS, complement components C3 & C5, ICAM1, and LTXα from T lymphocytes all induce IL6 and TNF expression from Kupffer cells. IL6 & TNFα prime hepatocytes after binding to IL6R and TNFαR. LTXα also acts directly on hepatocytes *via* the TNFαR. LSEC: Liver sinusoidal endothelial cell; IL6: Interleukin 6; IL6R: Interleukin 6 receptor; LPS: Lipopolysaccharide; TLR: Toll-like receptor; TNFα: TNF alpha; TNFαR: TNF alpha receptor; CtR: Complement receptor; ICAM1: Intercellular adhesion molecule 1; ICAM1R: Intercellular adhesion molecule 1 receptor; LTXα: Lymphotoxin alpha; LTXαR: Lymphotoxin alpha receptor.

of NFκB results in increased expression of both TNFα and IL6. TNFα may stimulate its own further expression in the Kupfer cell in an autocrine manner. IL6 binds IL6R on hepatocytes, producing activation of signal transducer and activator of transcription 3 (STAT3), which results in the transcription of multiple other genes which push hepatocyte from G0 to G1, thus priming the cell to be responsive to circulating growth factors.

Crucially, *in vivo*, infusion of the powerful complete mitogens EGF and HGF produces only modest hepatocyte proliferation, whereas marked hepatocyte proliferation is observed if EGF and HGF infusion is preceded by the priming effect of a single TNFα injection[196].

Consistent with this model the following events are observed in the minutes after PH: (1) TNFα and IL6 mRNA and protein increase immediately[197,198]; and (2) Activation of the transcription factors NFκB and STAT3[199,200]. Moreover, DNA replication in hepatocytes is blocked by TNFα antibodies[201], TNF receptor (TNFR)[57] and IL6[56] knockout mice show impaired liver regeneration, and liver regeneration in TNFR knockout mice is rescued with IL6 infusion[57].

Of note, highlighting the necessary caution needed before extrapolating between animal models, TNFα levels after PH differ between rats and mice, with higher levels in rats. Also, the model exemplifies the recurring theme of redundancy in the system with the TNFα knockout mice showing normal liver regeneration because of the ability of other ligands to bind the TNFR[202]. Similarly, the activation of STAT3 may be achieved by other cytokines than IL6, such as Stem Cell Factor[203] and Oncostatin [203].

Triggers to cytokine priming: The initial triggers to expression of the priming cytokines TNFα and IL6 after PH are doubtless numerous and not all identified, but at least 5 stimuli have been demonstrated.

Firstly, PH results in an immediate increase in portal venous pressure which causes a sheer stress on liver sinusoidal endothelial cells[156]. This physical stimulus has many consequences[159] which are discussed in more detail in section 2 on early events post hepatectomy, but which include the induction of IL6 expression in LSECs [164], thereby contributing to the priming of hepatocytes .

Secondly, another trigger to cytokine expression after PH is binding of LPS derived from gut bacteria and translocated to portal blood, to the TLR, and producing expression of IL6 and TNFα. The increase in portal pressure resulting from PH increases gut permeability and may therefore result in exposure of the remnant liver to higher concentrations of LPS[204]. Supporting the physiological relevance of this hypothesis, is the observation that rodents with germ free guts have impaired liver regeneration[205]. The effects of LPS on liver regeneration may not be limited to induction of the priming molecules IL6 and TNFα, but also producing an increase in secretion of hepatic mitogens including insulin, epidermal growth factor, and triiodo-

thyronine[204] (see "*Complete mitogens*" and "*Auxiliary mitogens*" on complete and auxiliary mitogens).

Thirdly, it is also known that binding of complement cascade components C3 and C5 to the complement receptors on Kupfer cells also triggers an NFκB dependant increase in both IL6 and TNF. Thus, complement activation resulting from physical injury to liver in PH may also contribute to the initiation of cytokine priming of hepatocytes. The significance of this mechanism is suggested by the finding that following PH, C3-5 knockout mice show diminished activation of NFκB and STAT3, decreased expression of TNFα and IL6 impaired liver regeneration[64].

Fourthly, it is observed that mice lacking the receptor intercellular cell adhesion molecule 1 (ICAM1) show diminished TNFα and IL6 expression and impaired liver regeneration after PH. It is thought that leucocytes, attracted to a liver injury site may mediate triggering of ICAM 1 on Kupfer cells, thus providing another stimulus to initiating the cytokine cascade[206].

Fifth, it is known that the TNFR may be activated not only by TNFα, but also by the protein lymphotoxin alpha (LTxα). This is markedly upregulated in intra-hepatic T lymphocytes after PH[207] and may thus allow T cells to contribute to initiation of the cytokine cascade by activation of TNFR on Kupffer cells. Consistent with this, mice lacking both TNFα and LTα show impaired liver regeneration[208]. Moreover, LTxα may act directly on hepatocytes.

Thus, having been primed by the initial injury triggered cytokine cascade, hepatocytes return to the G1 phase of the cell cycle where they are susceptible to stimulation by mitogens including growth factors, hormones and other biochemicals to accelerate the rate of proliferation.

The concept of mitogen hierarchy: A multitude of different hepatocyte mitogens have been identified which originate from a variety of different tissues, different cell types within a given tissue, acting *via* different receptors, or sometimes overlapping in their receptor binding, and producing a variety of different effects on the target hepatocyte. This complexity exemplifies a key feature of liver regeneration biology, which is the existence of high levels of redundancy, presumably an evolutionary outcome enabling the liver to cope meet the wide range of physical, biochemical and infectious injuries it may encounter.

Amongst this complexity however, as arisen the concept of a hierarchy amongst hepatic mitogens, classifying them as 'complete mitogens', 'auxiliary mitogens', and 'complex mitogens'[209]: (1) Complete mitogens cause proliferation of hepatocyte cultures in serum- free media, and, when injected into whole animals, cause liver enlargement and hepatocyte DNA synthesis. Moreover, ablation of both the MET and EGFR pathways leads to complete inhibition of liver regeneration. The complete mitogens are (a) Hepatocyte growth factor which binds to its receptor MET; and (b) Ligands of the EGFR: EGF, transforming growth factor-α (TGFα), heparin- binding EGF- like growth factor and amphiregulin; (2) Auxiliary mitogens do not cause hepatocyte proliferation in culture in serum free media, do not cause hepatocyte DNA synthesis and liver enlargement when injected *in vivo*, and ablation of their signalling pathways delays but does not abolish liver regeneration. The auxiliary mitogens are noradrenaline and the α1- adrenergic receptor, VEGF and its receptors (VEGFR1 and VEGFR2), bile acids, serotonin, insulin, and growth hormone; and (3) Complex mitogens are the third category and are much less well defined than complete or auxiliary mitogens, with pathways involving multiple overlapping extracellular signals, disruption of which delays but does not abolish liver regeneration. The complex mitogens are the proteins involved in the *Wnt*, *β-catenin*, *Hippo* and *Yap* pathways.

Complete mitogens

Hepatocyte growth factor: HGF was the first complete hepatic mitogen, identified in 1984 with the human homolog cloned in 1989[210]. Thus, HGF produces hepatocyte proliferation in serum free media *in vitro*, and liver enlargement when infused *in vivo*. HGF mediates its effect on hepatocytes by binding to its receptor MET, a receptor tyrosine kinase with wide ranging roles in diverse areas of cell biology including not only cell survival and proliferation[211], but also metabolism[212], growth and development[213]. MET signalling is dependent on the transcription factor CCAAT/enhancer-binding protein beta C/EBP beta[214], and Inhibition of MET signalling results in blocking of mitosis and increased expression of apoptosis genes after PH[215].

After PH, HGF is mobilised in a biphasic manner, first with the activation and recruitment of ECM bound inactive HGF in the immediate minutes after PH, and then secondly by secretion of newly expressed HGF in a second wave.

Thus, whilst HGF is bound in inactive form in the ECM in resting liver[216], ECM remodelling[187] resulting from PH results in activation of HGF with binding to hepatocytes and released into the circulation[217], which peaks 30-60 min after PH.

Thereafter, peaking at 24 h post PH[218], a second wave of HGF is observed, newly synthesized by LSEC and stellate cells in the liver, but also from extra-hepatic cells and tissues including platelets[219], lung[220], kidney, spleen[221], thyroid, brain, and salivary glands[221]. In spite of these multiple sites of HGF production, experiments using genetically altered mice showed that inhibiting HGF production specifically in LSECs resulted in impaired liver regeneration, suggesting that extra-hepatic HGF production cannot compensate for depletion of hepatic HGF production[222]. The factors that stimulate HGF expression in the second wave after its release from ECM include noradrenergic signals[223], insulin like growth factors[224].

Epidermal growth factor: Ligands of the EGFR make up the other known complete mitogens. EGF is one member of a family of 7 Ligands which bind a group of 4 receptors (EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3 and HER4/ErbB4)[225]. Of the 7 known ligands, the ones that relate to liver regeneration are epidermal EGF, transforming growth factor-alpha (TGFα), Amphiregulin (AR), and Heparin bound EGF-like growth factor (HB-EGF), with their role illustrated in Figure 2.

EGF is a complete mitogen and produces hepatocyte proliferation *in vitro* and *in vivo* when infused[226]. It is produced in many tissues[227], but the most relevant sites of production are the Brunner glands of the duodenum which provide a constant supply of EGF to the liver *via* the portal vein[228]. EGF production is increased by noradrenaline which is secreted during the physical stress of PH[229]. EGF is also produced in significant quantities in salivary glands, and sialadenectomised rats do show impaired liver regeneration after hepatectomy[230].

TGFα is produced by hepatocytes themselves during liver regeneration[231] and therefore functions as a mitogen in an autocrine or paracrine way[232]. TGFα knockout mice have no liver regeneration deficiency however, presumably as a result of the considerable redundancy in the EGFR signalling pathway[233].

Amphiregulin, like TGFα, is produced by hepatocytes. Its expression is in part regulated by inflammatory mediators providing a mechanism for its upregulation following PH. Its significance is suggested by the observation that AR knockout mice have impaired liver regeneration[234,235].

HB EGF is produced Kupffer cells and sinusoidal endothelial cells[236]. Its expression seems to be in part determined by the magnitude of liver resection, as it is increased in 2/3 PH but not 1/3 PH. Its physiological significance is emphasised by the fact that HBEGF transgenic mice[237] and HB EGF knockout mice[70] have accelerated and delayed liver regeneration, respectively.

In the midst of these multiple ligand binding events, EGFR activation peaks at 60 minutes post PH[238], and ablation of EGFR by antisense RNA impairs liver regeneration[239].

Auxiliary mitogens

Bile acids: Primary bile acids are synthesized in the liver by a multistep oxidative metabolism of cholesterol and secreted in bile. In the intestine, bile acids emulsify fats thus facilitating their digestion. Bile acids are metabolized by gut bacteria to produce secondary bile acids, and although some are lost through faecal excretion, a significant proportion are reabsorbed in the gut and recycled in the liver, in the entero-hepatic circulation[240].

Above a certain concentration, bile acids are toxic to liver and may induce apoptosis and necrosis, such that bile salt synthesis is tightly regulated by means of negative feedback loops involving bile acid receptors in the ileum[241]. At non-toxic concentrations however, bile acids play an important part in regulation of liver regeneration. Both the negative feedback controls and liver regenerative roles are mediated by bile acid receptors which comprise the extracellular TGR5 receptor (TGR5) on Kupffer cells [242], and intracellular Farnesoid X receptor (FXR) within hepatocytes[243].

Bile acids were first investigated as candidate factors for controlling liver regeneration in part because of their exclusively hepatic synthesis, offering the potential for a feedback loop hepatostatic mechanism. Thus, dietary bile acid supplementation was found to produce hepatomegaly in mice with non-injured livers, and increase liver regeneration after PH, in an effect that was dependent on the FXR. Conversely, bile acid sequestering agents resulted in impaired liver regeneration[243].

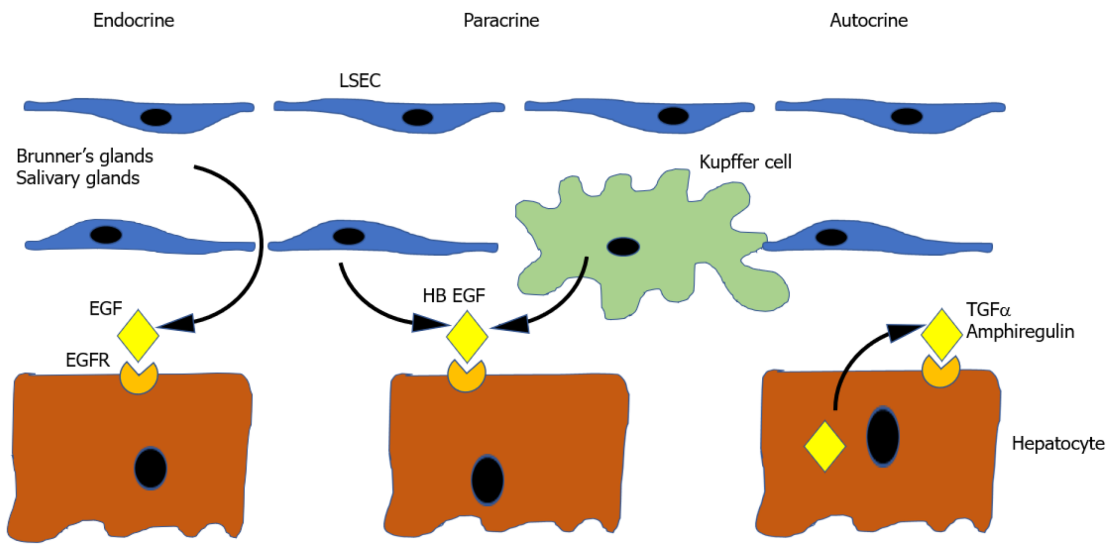

Figure 2 Summary of ligand binding to epidermal growth factor receptor in liver regeneration. Endocrine EGFR signalling by EGF from Brunner's glands and salivary glands. Paracrine EGFR signalling by HB EGF from LSEC and Kupffer cells, autocrine EGFR signalling by amphiregulin and TGFα from hepatocytes. LSEC: Liver sinusoidal endothelial cell; EGF: Epidermal growth factor; EGFR: Epidermal growth factor receptor; HB EGF: Heparin bound EGF-like growth factor; TGFα: Transforming growth factor α.

Furthermore, genetically engineered bile salt deficient mice also show impaired liver regeneration after PH[244], and rats having undergone PH with biliary fistula also show impaired liver regeneration, which can be rescued by intestinal delivery of bile acids[245].

After PH, serum bile acid concentration increases in blood within minutes, peaks at 24 h, and diminishes again by 48 h. The mechanism of this serum bile acid increase is not fully understood, but may involve neurological pathways activated by PH related changes in portal pressure[246], consistent with the observation that bile acid increase is also seen after portal vein embolization[247].

The binding of bile acids to the FXR stimulates activation of transcription factor Forkhead box M1b (FoxM1b), an injury-induced transcription factor that promotes cell cycle progression[248]. In addition, bile acids also contribute to liver regeneration by binding extra-hepatic FXR situated in the ileum, resulting in expression of fibroblast growth factor (Fgf15/FGF19). Fgf15/FGF19, which then binds it receptor FGFR4[249] on hepatocytes, stimulating cell cycle progression[241]. In comparison to the FXR receptor, the role of bile acid binding to the TGR5 receptor on Kupffer cells is less well understood, but is clearly important as TGR5 knockout mice show impaired liver regeneration after PH, as well as severe hepatic necrosis[250].

Thus, bile acids have an important role in the control of liver regeneration and may contribute to the post liver injury hepatostat.

Noradrenaline: Noradrenaline secretion increases following PH[251] and is produced by the adrenal medulla, sympathetic neurons, as well as by hepatic stellate cells.

Noradrenalin not only stimulates the production of EGF (from Brunner's glands) and HGF from fibroblasts, but also augments their mitogenic effect[252], and activates the proliferation associated STAT3 pathway[253], whilst reducing the mito-inhibitory effects of TGFβ[254]. Thus, α1 receptor blockade, and also hepatic sympathectomy significantly delays liver regeneration after PH[251]. Noradrenaline may also stimulate liver regeneration by activating WNT and β-catenin pathways *via* β-adrenergic receptors[255].

Serotonin: Serotonin is a neurotransmitter stored by platelets and which has a role in the control of inflammation. Mice with absent platelets or lacking tryptophan hydroxylase 1 (a key enzyme in serotonin synthesis) show significantly delayed liver regeneration after PH[256], which is rescued by serotonin infusion. Moreover, serotonin agonist produces LSEC fenestration changes, and a VEGF dependent increase in hepatocyte proliferation[257]. Serotonin may also act *via* the Hippo proliferative pathway[258]. Although serotonin deficient mice show significantly impaired liver regeneration, serotonin exemplifies the need for caution in assuming that the results of one animal model may be extrapolated to others, as it is noted that rats

lacking the serotonin transporter which are unable to store serotonin in platelets do not show any liver regeneration impairment after PH[259].

Insulin: Insulin is produced by the beta cells of the pancreas, and was one of the earliest identified hepatic mitogens, having been found to prevent liver atrophy when infused directly into the liver *via* the portal stump in dogs having undergone portocaval shunt[11,13]. Although insulin is not a complete mitogen in that it does not induce hepatocyte proliferation *in vitro*, its presence is essential for hepatocyte survival in culture[260] and is essential for the effects of complete mitogens EGF and HGF *in vitro*[261].

The paradox that insulin is not a complete mitogen *in vitro*, but able to prevent liver atrophy after portal diversion is not fully understood but may be partly explained by interactions of the insulin receptor with EGFR and MET, thus triggering those proliferative pathways[212].

Growth hormone and insulin-like growth factor: Growth hormone is synthesized in the pituitary gland and has widespread growth-related roles in many tissues[262]. The effects of GH can be mediated directly *via* the GH receptor, or indirectly by insulin-like growth factor (IGF), which is synthesized by hepatocytes in response to GH and secreted into the circulation bound to IGF binding protein (IGFBP). Whilst hepatocytes do not have IGF receptors[263], Kupffer cells and stellate cells do[264], allowing a possible paracrine role for IGF in the liver. GH may also act directly on hepatocytes by upregulating the EGFR[265] and also stimulating activity of the EGFR in cross-talk with the GHR[266]. Consistent with this, in the rat model, exogenous dietary or infused GH enhances liver regeneration after PH, and mice lacking IGFBP show impaired liver regeneration after PH[63].

In terms of the physiological relative importance of these pathways, Pennisi *et al* [267] showed that GH lacking mice showed the greatest impairment to liver regeneration, with less marked liver regeneration impairment seen in IGF and IGFBP lacking mice, suggesting that whilst both direct and indirect GH actions impact on liver regeneration, the direct effect of GH is more significant that IGF mediated effect.

Thyroid hormone: The thyroid hormones Triiodothyronine (T3) and thyroxine (T4) are produced in the follicular cells of the thyroid gland, and have extensive roles in carbohydrate, protein, and lipid metabolism, regulation of metabolic rate, oxygen consumption, thermal regulation, muscle function, and roles in tissue growth and development[268].

In terms of liver regeneration, thyroid hormones have been shown to act as incomplete mitogens with impaired liver regeneration seen in thyroid receptor knockout mice[269], and conversely accelerated liver regeneration in T3 treated rats after PH[270].

In terms of molecular mechanisms of action in promoting liver regeneration, the thyroid hormones do not act *via* the NFκB or STAT3 pathways which are typically activated by the complete mitogens. Rather, thyroid hormones mediate hepatocyte proliferation by a number of pathways including (1) Increase in expression of transcription factors of the E2F family, which accelerates the transition of hepatocytes from G1 to S phase[271]; (2) Increased expression of cell cycle promotion genes Cyclins A, D1, and E, and diminished expression of their inhibitors[272]; (3) Decreased levels of p53 and p73 (tumour suppressor proteins involved in growth arrest and apoptosis) [269]; and (4) Activation of the *Wnt/b-catenin* signalling pathway[273].

VEGF: The VEGF family of growth factors comprises a group of at least 6 isoforms (VEGF A, B, C, D, E, F), which bind to the 3 different receptors (VEGFR 1, 2, 3), with roles in cell proliferation, migration, metabolism, vasodilation, blood vessel formation and remodelling[274]. Though not directly mitogenic on hepatocytes directly, VEGF plays a central role in liver regeneration in several ways, including the orchestration of proliferation of LSECs, and inducing the LSEC population to produce key hepatocyte mitogens including HGF[175,275].

In a rat 70% PH model, Bockhorn *et al*[276] showed that blocking VEGF signalling with anti VEGF antibodies almost completely suppressed hepatic proliferation in the first 24 h after surgery, and conversely that exogenous VEGF promoted hepatocyte proliferation, suggesting a physiologically relevant role for VEGF in the early stages of liver regeneration.

Complex mitogens

WNT/β-catenin signalling pathway: The WNT family of genes are named after the

gene responsible for the Wingless-type phenotype in *Drosophila melanogaster*, and int-1 (a target for insertional activation of mouse mammary tumour virus, and a secretory glycolipoprotein-encoding gene which is regarded as the prototype for several mammalian genes)[277]. The resulting glycolipoproteins participate in several fundamental signalling events, which influence cell proliferation and tissue homeostasis[278].

β-catenin is a protein encoded by the CTNNB1 and is a subunit of the cadherin protein complex which acts as an intracellular signal transducer in the context of WNT signalling, but which also interacts with a variety of transcription factors such as T-cell factor and hypoxia-inducible factor 1α[279]. β-catenin plays an important role in human embryogenesis, including liver development[280]. It is widely expressed in the adult liver and is always active in the pericentral region. Usually bound to a multiprotein degradation complex, it can be activated by several pathways, including WNT.

The WNT/β-catenin pathway has been shown to be active during liver regeneration, contributing towards mass and functional recovery from a the very early stages of liver injury[281]. β-catenin is in fact detected in rat hepatocyte nuclei within 5 minutes of partial hepatectomy[282]. Upon WNT binding to its receptor (Frizzled), β-catenin translocates to the nucleus, where it promotes the expression of key genes, such as high-level controllers of transcription like *c-myc* and cell cycle regulating genes like cyclin D1[279].

In the normal liver, β-catenin regulates the expression of genes in pericentral hepatocytes and influences hepatic lobular zonation[279] and is involved in cell-cell adhesion[280]. Additionally, it implicated in a variety of diseased liver states, although the exact mechanisms remain incompletely understood. Specifically, β-catenin appears to be involved in the development of NASH, partly by binding to TCF4 and HNF4α, thus regulating hepatic gluconeogenesis and lipogenesis. In hepatic fibrosis, the literature is currently conflicting regarding the role of WNT/β-catenin signalling. Nevertheless, evidence is accumulating to show that this signalling pathway is activating during hepatic stellate cell activation and fibrosis, and that WNT blockade is associated with an antifibrotic effect[279].

This pathway has also been identified in hepatic neoplasia. In focal nodular hyperplasia (FNH), glutathione synthetase (the expression of which is regulated by WNT/β-catenin signalling) stains FNH, which may be of diagnostic value[279]. Other relevant neoplastic processes include hepatocellular adenoma both with and without the presence of CTNNB1 mutations, HCC where mutations may lead to autonomous WNT-mediated activation of β-catenin, and hepatoblastoma, where 90% of tumours are associated with CTNNB1 mutations[279].

Hedgehog signalling pathway: Emerging evidence in the literature has shown the importance of the activation of the Hedgehog (Hh) pathway in the context of liver regeneration. Hh is a protein produced as a 45-kDa precursor that undergoes proteolytic processing in the endoplasmic reticulum[283]. The Hh pathway is a highly complex signalling cascade, which may be summarised in four fundamental components: (1) The ligand Hedgehog; (2) The receptor Patched (Patch); (3) The signal transducer Smoothened (Smo); and (4) The effector transcription factor, Gli. Components of the Hh pathway concentrate in the Primary cilia and a complex Primary cilium trafficking system regulates the interaction of Hh pathway components to enhance, or block, the Hh-initiated signal[284-286].

Previous work in adult rodents has demonstrated that Hh ligand expression increases transiently but significantly following partial hepatectomy[287]. Furthermore, inhibiting Hh pathway induction with a direct pharmacologic antagonist of Smo was found to decrease both recovery of liver volume and overall survival[288]. Evidence in the literature suggests that, mice subjected to portal vein ligation (a procedure commonly done in humans to allow the remnant liver to enlarge prior to hepatectomy) with simultaneous administration of systemic Hh, performed as well as mice submitted to ALPPS, supporting the evidence that Hh signaling plays a major role in promoting liver regeneration[289]. Further evidence suggests that the extracellular matrix of the healthy adult liver, the proteoglycan glypican-3 binds normally to Hh to prevent Hh from binding to Patch in order to constrain activation of the Hh pathway[78].

The evidence above highlights the role of the Hh pathway in post-hepatectomy liver regeneration. Further translational studies are required in order to explore the role of administering a recombinant form of Hh in the pre-operative setting in patients undergoing major hepatectomy who are potentially at risk of liver insufficiency.

Hippo Yap signalling pathway: The Hippo signalling pathway was originally identified in *Drosophila melanogaster* and its components have mammalian homologs [290]. The Hippo signalling pathway exerts a controlling influence on organ size by regulatory effects on cell proliferation, apoptosis, and stem cell self-renewal[291].

The pathway consists of a series of protein kinases, activation of which results in the phosphorylation of yes-associated protein (YAP), thus preventing its translocation to the nucleus. In the nucleus YAP interacts with a family of transcription enhancer factors. This family of nuclear proteins are involved in the modulation and regulation of multiple genes involved in promoting cell proliferation[292-294].

Multiple signals may activate Hippo signalling, including mechanical stimuli and cell attachment. Thus, in situations of high cell density, activation of Hippo signalling leads to the inhibition of YAP nuclear translocation, and thereby a break on cell proliferation[295]. Conversely, decreased Hippo pathway signalling allows YAP mediated pro-proliferation signals and is associated with an increase in organ size through excessive proliferation and inhibition of apoptosis[292]. Consistent with this, and suggesting its physiological importance, mice with liver specific YAP deletions show significant impairment in liver regeneration[296].

However, the Hippo-YAP signalling pathway is the final step of multiple opposing signalling pathways that contribute to liver regeneration and repair and the conflicting nature of these signals makes the study and understanding of the role of this pathway challenging[209].

Intracellular signalling pathways

The complexity inherent in the multitude of extracellular molecules implicated in the control of liver regeneration is reflected by a similarly complex array of intracellular signalling pathways which transmit the effect of ligand-receptor binding to the nucleus to activate effector genes.

In the same way that there is much redundancy in growth factor function extracellularly, there is also much overlap in the intracellular pathways, probably reflecting an evolutionary mechanism to safeguard against failure of any one individual pathway.

The complexity of each pathway, the activation of different pathways by diverse ligands, and the intracellular cross-talk between pathways makes it difficult to assign quantitative importance to any one pathway. Nevertheless, the section describes the main recognized intracellular pathways relating to liver regeneration. A full account of this area is beyond the scope of this review, but the summary below is intended to give a general overview and an impression of the ramifying complexity of the processes involved.

Figure 3A shows the pathways separately in summary form. Figure 3B shows the overlap in ligand binding, and Figure 3C provides an impression of the cross-talk between the various pathways.

Ras/Raf/MEK/ERK pathway: The Ras/Raf/MEK/ERK Pathway is triggered by binding of ligands to receptor tyrosine kinase receptors, which triggers autophosphorylation of tyrosine residues on the intracellular aspect of the receptor, resulting in the sequential activation of downstream components, ultimately controlling the expression of multiple growth controlling genes including high level 'master genes' such as *c-myc, c-fos, and c-jun*[297]. The first molecule, RAS, once activated, can activate multiple different signalling intracellular pathways including not only the Raf/MEK/ERK pathway, but also the MEKK/SEK/JNK pathway, and pathways involving NFκB[298,299].

In liver regeneration, the growth factor which activate this pathway include HGF, the EGFR ligand family members, fibroblast growth factor, and VEGF[299].

Phosphatidylinositol 3'-kinase/AKT kinase (also known as protein kinase B)/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway: The PI3K/ Akt/mTOR is a ubiquitous pathway is involved in the regulation of fundamental physiological processes including transcription, apoptosis, cell cycle progression, and translation [299]. The pathway is activated by binding of ligands to receptor tyrosine kinases or G-protein-coupled receptors[300], ultimately promoting cell growth, proliferation, survival [301], and malignancy when dysregulated[302]. In the context of liver regeneration, the main growth factors activating the PI3K/Akt/mTOR pathway include TNF-α, IL-6, HGF, EGF, and transforming growth factor (TGF)-α[303] (Figure 3B and C).

Janus Kinase pathway: Activation of the Janus Kinase (JAK) kinase pathway promotes cell proliferation, differentiation, migration, growth. Like RTKs, JAKs activate by

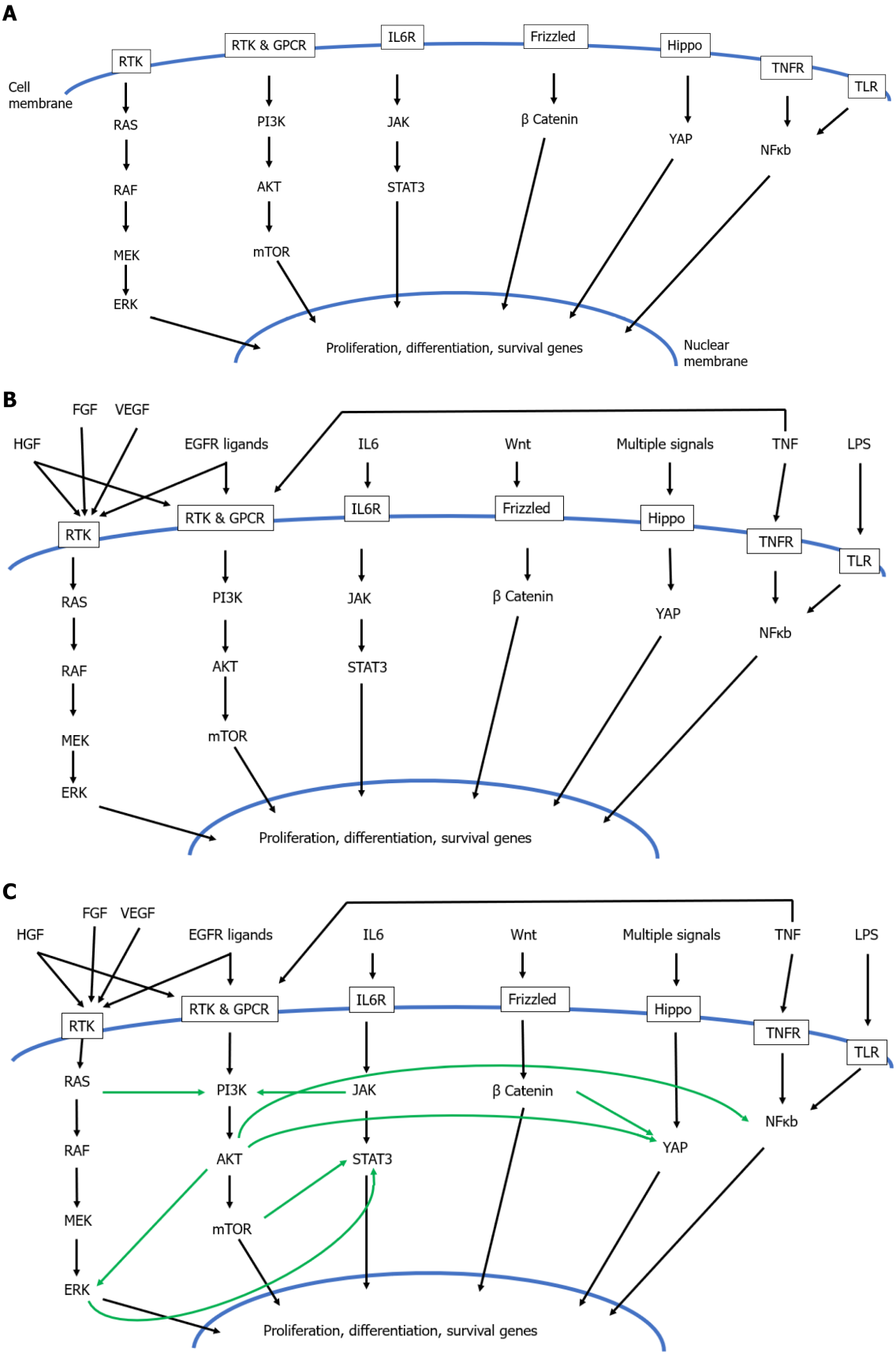

Figure 3 Intracellular signal transduction map. A: Intracellular signal transduction in liver regeneration; B: Ligand overlap and receptor binding redundancy; C: Intracellular cross talk between signalling pathways. HGF: Hepatocyte growth factor; FGF: Fibroblast growth factor; VEGF: Vascular endothelial cell growth factor; EGFR: Epidermal growth factor receptor; IL6: Interleukin 6; TNF: Tumour necrosis factor; LPS: Lipopolysaccharide; RTK: Receptor tyrosine kinase family (including HGF receptor, FGF receptor, VEGF receptor, EGF receptor); GPCR: G protein coupled receptor; IL6R: Interleukin 6 receptor; TNFR: Tumour necrosis factor receptor; TLR: Toll like receptor; RAS/RAF/MEK/ERK: signalling components downstream of receptor tyrosine kinase; PI3K: Phosphatidylinositol 3'-kinase; AKT: Akt kinase

(also known as protein kinase B); mTOR: Mammalian target of rapamycin; JAK: Janus Kinase; STAT3: Signal Transducer And Activator Of Transcription 3; YAP: Yes-associated protein; NFκb: Nuclear factor kappa B.

autophosphorylation. STAT3 is the key downstream messenger which translocates to the nucleus and functions as a high level transcription factor in liver regeneration with resultant induction of gene expression including cytokines[304]. In the context of liver regeneration, this pathway is primarily activated by IL-6 and its receptor, IL-6R.

Interestingly, following PH, a circulating (rather than membrane bound) form of the IL6R cleaved by matrix metalloproteases appears to have a key role in initiating liver regeneration[86].

NF-κB pathway: In priming of hepatocytes, Kupffer cells are induced to secrete TNF-α and IL-6 after stimulation by a variety of stimuli (see section on hepatocyte priming) including complement factors and LPS from the gut. This action of Kupffer cells is mediated by the NF-κB signaling pathway. Once activated, NF-κB migrates to the nucleus, where it promotes the further expression of TNF, IL-6, and VEGF[305].

WNT/β-Catenin pathway: The *Wnt/β-catenin* pathway regulates processes including cell proliferation, and tissue morphology[282]. Upon WNT binding to its receptor (Frizzled), β-catenin translocates to the nucleus, where it promotes the expression of key genes, such as high-level controllers of transcription like *c-myc* and cell cycle regulating genes like cyclin D1[279].

***Hippo* pathway:** The *Hippo* signalling pathway is involved in cell proliferation, apoptosis, and stem cell self-renewal[291], and may have a key role in the ending of hepatocyte proliferation after regeneration[295]. YAP is a key downstream effector of the *Hippo* pathway, which translocates to the nucleus, once activated to promotes expression of target genes. The pathway is activated by numerous factors including organ size, cell attachment, mechanical stress, hormones, growth factors[295], as well as vascular shear stress[306].

Cross-talk between pathways: Mirroring the redundancy and overlap in extracellular growth factors and receptor binding, there exists a complex crosstalk between pathways. Just a few examples of the known positive feedback interactions are shown in Figure 3C, which, albeit incomplete and without including negative feedback interactions, provides an impression of the intricacy of intracellular interactions between pathways[297]. Thus, Ras interacts with phosphatidylinositol 3′-kinase (PI3K)[307]. The NF-κB pathway cross-talks with the PI3K/Akt/mTOR, pathways[308]. β-catenin interacts with the Hippo signalling pathways[195]. YAP cross-talks with PI3K/Akt pathway[309].

SECTION 4: THE CONTRIBUTION OF NON-PARENCHYMAL CELLS TO LIVER REGENERATION

The proliferation of hepatocytes in liver regeneration is in critical ways dependant on the role of non-parenchymal cells (Kupffer cells, stellate cells, and liver sinusoidal endothelial cells). Conversely, proliferating hepatocytes provide many growth factors that elicit non parenchymal cell proliferation, thus suggesting an interdependence allowing proportionate expansion of each cell type to produce liver tissue containing all key constituents. Thus, proliferating hepatocytes produceVEGF and angiopoietins 1 and 2 (LSECs mitogen), TGFα (LSECs and stellate cell mitogen), fibroblast growth factor 1 (FGF1) and FGF2 (HSC and LSEC mitogen), and granulocyte–macrophage colonystimulating factor (GMCSF) (Kupffer cells mitogen)[191,310,311]. In this section we examine in more detail the part played by non-parenchymal cells in liver regeneration.

Liver sinusoidal endothelial cells

LSECs have a key role in the immediate events that trigger the onset of hepatocyte proliferation after PH. Thus, shear stress resulting from portal pressure changes after PH induces the expression of the hepatocyte priming IL6[164].

Once liver regeneration is initiated, hepatocytes form clusters which are initially avascular. The production of VEGF, and angiopoietin 1 & 2 stimulates endothelial cells to migrate into the avascular structures, proliferate, differentiate to a liver sinusoidal phenotype[312]. LSECs also produce VEGF[313], as well as the potent mitogen HGF, and NO[160,162] which increases hepatocyte sensitivity to HGF[163].

In the rat hepatectomy model, liver sinusoidal cell repopulation is not only achieved by resident LSECs, but also by recruitment of LSEC progenitors in the bone marrow which, under the influence of VEGF, undergo proliferation, migration into the bloodstream, and engraftment in the liver, where they contribute a significant proportion of the total HGF production.

Of note, the importance of bone marrow derived LSECs in this model is emphasised by the fact that bone marrow ablation by irradiation abolishes liver regeneration, which can be rescued by exogenous infusion of LSECs[314,315]. Thus, LSECs play a key role, not only in allowing the vasculature of the liver to keep pace with regenerating hepatocytes, but also in providing the very growth factors (such as HGF) that allow hepatocyte to proliferate.

Stellate cells

Stellate cells are situated in the Space of Disse between LSECs and hepatocytes [316], and though representing only approximately 8% of cells in the liver, have multiple long cytoplasmic projections[317] which contact hepatocytes, LSECs, and Kupffer cells, allowing a central role in intercellular signalling in part by production of growth factors such as HGF and VEGF. Stellate cells also exhibit cellular contraction, permitting the control of sinusoidal blood flow[318], and have a key role in the regulation of ECM, both in its production and degradation[319].

After hepatic injury, HSC become activated myofibroblast-like cells. In the midst of liver regeneration, the initiation, perpetuation and resolution of HSC activation only adds further complexity, in processes which are poorly understood[320], but which ultimately result in the laying down of ECM to provide the vital framework on which regeneration may proceed[321].

The triggering of HSC activation is multifactorial, but includes the secretion by hepatocytes of growth factors such as FGF1 & 2, and PDGF, the latter being a potent mitogen and chemo-attractant for HSCs[322], and highlighting an example of interdependent complex paracrine stimulation (with HSCs producing the hepatocyte mitogen HGF and hepatocytes producing the HSC mitogen PDGF).

Following activation, HSC production of HGF increases, in a mechanism dependant on the neurotrophin receptor P75NTR[323], and its downstream mediator Rho[324]. Activated HSCs also produce Noradrenaline, which enhances HGF production by mesenchymal cells[223] and production of EGF from Brunner's glands[229]. HSCs interact directly with LSECs to stabilise and remodel sinusoids[325], *via* combined actions of PDGF, TGF-β1, FGF, VEGF, and angiopoietin. HSC are the principal cell source of ECM constituent production, and of ECM remodelling control by expression of matrix metalloproteases, thus providing the scaffold in which liver cells can regenerate[326].

The importance of HSC activation in liver regeneration is suggested by the observation that following ablation of HCS activation, liver regeneration is markedly impaired in both the mouse acetaminophen[327] and rat acetyl amino fluorene[328] models of liver injury with much reduced proliferation of hepatocytes and oval cells respectively, and with rescue of liver regeneration by infusion of medium conditioned by HSC[329].

Kupffer cells

Amongst the different resident intrahepatic macrophages, Kupffer cells are the predominant type, and originate from erythromyeloid progenitors in the foetal liver [330]. In the homeostatic situation, Kupffer cells have wide-ranging roles in (1) Clearance of cellular debris in blood[331]; (2) Maintenance of iron homeostasis *via* phagocytosis of red blood cells[332]; (3) Regulation of cholesterol homeostasis[333]; (4) Antimicrobial defence[334]; and (5) Promotion of immunological tolerance[335].

Kupffer cells have a limited half-life (of approximately 12 d in mice)[336]. Their maintenance is achieved by self-replenishment in the healthy liver[337], but is to an extent dependant on extra-hepatic progenitors in the case of liver injury[338].

There is evidence that replenishment of Kupffer cells following injury may be achieved by engraftment and differentiation of monocyte derived macrophages into a Kupffer cell phenotype, in a manner controlled by HCS and LSEC, highlighting once again the complex cross-talk between the non-parenchymal cell types[339]. The monocyte derived macrophage precursors may originate from within the liver, but

also from the peritoneum[340] and spleen[341].

Kupffer cells are strategically placed with access to the sinusoidal lumen and space of Disse, and carry multiple surface receptors to injury related molecules (the Pattern Recognition Receptors), suggesting a key role for Kupffer cells as sensors of hepatic injury[342].

Thus, Kupffer cells are able to detect, and become activated by: (1) DAMPs, *e.g.*, mitochondrial DNA (mtDNA), and ATP, from damaged hepatocytes; (2) Pathogen-associated molecular patterns (PAMPs)[343]; (3) Hypoxic liver environment[344]; and (4) Extracellular vesicles secreted from various cells containing proinflammatory stimuli[345].

The activation of Kupffer cells by any of the above stimuli results in the secretion a wide range of bioactive molecules including chemokines such as C chemokine 2 which attract inflammatory and immune response cells to the injury site[346], and proinflammatory cytokines such as IL6 and TNFα which prime hepatocytes out of G0 phase an also activate HSCs[347].

Thus, Kupffer cells have a central role in the initiation and orchestration of liver regeneration, and their importance is suggested by the observation that depletion of macrophages[206] or inhibition of monocyte[348] recruitment results in impaired liver regeneration following PH.

SECTION 5: THE 'ALTERNATIVE PATHWAYS' OF LIVER REGENERATION

The liver regenerative response varies not only according to the nature of the injury, but also its magnitude and the status of the underlying liver parenchyma. These different contexts dictate how regenerating cells behave, but also the recruitment of different types of cells to accomplish the task. Current knowledge on the mechanisms of liver regeneration is largely derived from experimental models involving 2/3 PH in rodents[195], where "standard" liver regeneration occurs. This involves the proliferation of hepatocytes and cholangiocytes from homotypic precursors[209] and is addressed in the first subsection below. In the second subsection, the "alternative" liver regeneration pathways, which involve liver progenitor cells (LPCs) and transdifferentiation, will be examined. As seen in other aspects of liver regeneration, the mechanisms outlined below are subject to ongoing scientific scrutiny and are currently incompletely understood.

Hepatocyte response heterogeneity after PH

Liver regeneration after PH is achieved in different ways according to the magnitude of the liver resection. Thus regeneration after 1/3 PH is achieved principally by hypertrophy, with few cell divisions[349]. In contrast, during liver regeneration after resections larger than 1/3PH, although hypertrophy precedes hyperplasia, hyperplasia occurs increasingly as well, such that hypertrophy and hyperplasia contribute equally to liver regeneration in 70% PH. Moreover, during the hyperplastic response, although the majority of hepatocytes entered S phase of the cell cycle, not all undergo actual cell division, and the known significant number of polyploid hepatocytes[350] are shown to undergo division to produce mononuclear hepatocytes.

Thus, hepatocyte behaviour during liver regeneration is not uniform. Hepatocytes within a liver lobule are not equivalent and show functional heterogeneity. The liver lobule may be separated into 3 zones: Zone 1 (periportal) hepatocytes are in the vicinity of the portal triad, zone 3 (perivenular) hepatocytes are situated near the central vein, and zone 2 (pericentral) hepatocytes reside between zones 1 and 2. Metabolically speaking, zone 1 hepatocytes carry out gluconeogenesis and b-oxidation, in contrast to glycolysis, lipogenesis, and detoxification performed by zone 3 hepatocytes[195].

Moreover, there is some evidence that there is heterogeneity in baseline hepatocyte turnover during homeostasis, with a population of cells in zone 3 replenishing the lobule population, albeit slowly[351].

Hepatocyte proliferation heterogeneity is also apparent in the context of the regenerative response. In the context of true proliferative response after PH, lineage experiments have identified a population of hepatocytes in the periportal region zone 1 which appear to have greater proliferative potential. These are referred to as 'hybrid hepatocytes 'in that in addition to hepatocyte markers, they express progenitor cell genes and biliary transcription factors[352]. There appears to be further heterogeneity in proliferative response in that hepatocytes in proximity to LSEC proliferate faster than ones which are more distant[175].

Facultative stem cells and transdifferentiation pathways

The alternative liver regenerative pathways are characterized by deviation from the phenotypic fidelity in which hepatocytes or cholangiocytes proliferate to produce more of the same cell type. In this "alternative" context, liver epithelial cells (*i.e.*, hepatocytes and cholangiocytes) can operate as facultative stem cells for one another in conditions where regeneration of one or other is impaired[209,353], presumably as a rescue mechanism. This mechanism appears beneficial from an evolutionary standpoint, is plausible from a developmental biology perspective given that both hepatocytes and cholangiocytes are derived from hepatoblasts[354], and has been demonstrated in previous studies[81,355-357]. Nevertheless, there is controversy in the field regarding the *in vivo* capability, conditions, and extent to which liver epithelial cells can transdifferentiate and achieve regeneration.

The term "LPCs" is seen in the literature, yet such cells have not been identified on microscopy or tissue dissociation of liver lobules in the resting state and are thought to possibly arise from the transdifferentiation of hepatocytes and cholangiocytes[353] as mentioned above. A central event in this process is the "ductular reaction", which occurs when hepatocyte proliferation is suppressed, thus leading to the expansion of progenitor cells[209] and which can be observed both in acute and chronic liver disease models, typically after extensive hepatocyte injury.

In conditions such as fulminant hepatic failure, some liver epithelial cells demonstrate an overlapping set of biomarkers (*e.g.*, cholangiocytes may express "hepatocytic" biomarkers such as HNF4, albumin, and HEPPAR3)[353]. The extensive necrosis and apoptosis characteristic of fulminant hepatic failure is thought to pivot liver regeneration towards LPCs, as reflected in elevated α-fetoprotein levels[358] and as demonstrated by histological findings of "regenerative clusters", which consist of atypical ductules lined by cells exhibiting a combination of cholangiocyte and hepatocyte biomarkers[209].

In the context of PH, once a certain resection threshold is exceeded (*e.g.*, > 80%), adequate liver regeneration cannot be achieved by the relatively small number of remaining hepatocytes, and the alternative pathway is thus activated. In this process, biliary epithelial cells (cholangiocytes) de-differentiate into progenitor cells and then re-differentiate into hepatocytes in order to repopulate the liver[195]. As described in the rat models subsection above, Evarts *et al*[359] demonstrated that administration of 2-AAF to rats which had undergone PH was associated with differentiation of oval cells (a putative hepatic progenitor cell) to hepatocytes. However, this model did not allow for genomic-based cell lineage tagging[209].

More recently, Lu *et al*[81] induced widespread hepatocyte injury in mice through Mdm 2 deletion, which results in p53 upregulation with p53-induced hepatocyte death and senescence. The authors found that widespread hepatocyte injury was associated with a ductular reaction, whereby hepatocyte progenitor cell populations expanded and where bromodeoxyuridine-positive hepatocyte progenitor cells were often closely associated with bromodeoxyuridine-positive hepatocytes, thus suggesting that (in this context) hepatocytes arise from progenitor cells[81]. In a zebrafish model, Choi *et al*[355] found that after severe hepatocyte depletion, biliary epithelial cells de-differentiated into hepatoblast-like cells and then differentiated into highly proliferative hepatocytes, thus leading to liver regeneration.

Although the above studies focused on transdifferentiation from cholangiocytes to hepatocytes, the inverse has also been demonstrated in a murine model of Alagille syndrome (a human genetic condition associated with biliary underdevelopment). In this study, Schaub *et al*[360] found that hepatocytes converted to mature cholangiocytes that were effective in supporting biliary drainage and remained so after cholestasis resolved, in a TGFβ signalling mediated process. This persistent phenotypic change is distinct from the reversible conversion of human or murine hepatocytes to progenitor cells seen in other studies[361] and which may more accurately be described as "metaplasia" rather than "transdifferentiation". In their chimeric liver rat model, Michalopoulos *et al*[362] injected dipeptidyl peptidase IV (DPPIV)-positive rat hepatocytes into DPPIV-negative rats which then underwent partial hepatectomy and bile duct ligation, with or without additional biliary injury by methylene diamiline (DAPM) administration. On animal sacrifice after 30 d, the authors found that ductules exhibited the DPPIV marker, and that this was enhanced 36-fold in rats with additional DAPM-mediated biliary toxicity.

Evidence in support of liver epithelial cell transdifferentiation for regeneration is accumulating, yet several areas of controversy remain to be resolved. In addition to the findings described above, self-renewing facultative stem cells have been located in peribiliary glands and liver progenitor cells of bipotential differentiation capacity have been located in association with the canals of Hering. However, their role in liver

regeneration has been disputed[195]. Also, it remains unclear whether all mature hepatocytes are capable of dedifferentiation to progenitor cells, or whether this is only possible in a subset of cells[195]. Finally, various signalling pathways (*e.g.*, YAP, Rho kinase, TGF-β, glycogen synthase kinase 3) have been implicated in hepatocyte dedifferentiation in animal models[195], yet their role with respect to human hepatocytes remains to be clarified.

SECTION 6: THE INFLUENCE OF UNDERLYING LIVER DISEASE ON LIVER REGENERATION

The processes and mechanisms of liver regeneration are not only influenced by the magnitude and type of injury, but also by the status of the underlying liver parenchyma prior to injury. Although the PH model has contributed much information relating to events relating to regeneration of normal liver, significant differences come to light when the underlying liver is diseased. This section describes the ways in which liver regeneration is altered in the instances of age-related liver impairment, acute liver injury, hepatic steatosis, fibrosis, and cirrhosis.

Age-related liver impairment

Although the adult liver retains regenerative capacity throughout life, this is reduced in old age[363], through several suggested mechanisms. FoxM1B is a transcription factor expressed during embryonal development and also in liver regeneration[364]. Its expression is diminished in aged mice, whose liver regeneration can be rescued through its transgenic overexpression[365]. Age-related liver regeneration impairment may also be mediated by changes in the expression and function of cell cycle affecting genes such as CCAAT/enhancer-binding protein (C/EBP)α, which is an inhibitor of Cyclin D[366]. Budding uninhibited by benzimidazole-related 1 (BubR1) is involved in the control of mitosis and is found to be diminished in old age. Genetically manipulated mice expressing low levels of BubR1 show impaired liver regeneration [367].

In addition to these mechanisms, Conboy *et al*[368] and Liu *et al*[369] have demonstrated in parabiotic experiments that the blood or plasma of young mice partially rescues the liver regenerative compromise seen in old mice, suggesting the presence of currently unidentified circulating factors, in work reminiscent of the early experimental approaches used to demonstrate the existence of portal mitogens.

Acute liver injury

Severe acute liver injury may result from a variety of insults including viral infection (*e.g.*, Hepatitis A, B, C), poisoning (*e.g.*, paracetamol) or auto-immune disease. Though different in nature, these diverse insults nevertheless have the common feature of causing significant necrosis and apoptosis, in the midst of which regeneration must happen.

Specific models provide some mechanistic information. For example, the mouse model of paracetamol injury suggests that beyond a threshold of injury, regeneration fails, and that this is associated with failure of β catenin activation, consistent with the correlation of β-catenin activation and regeneration seen in patients[370].

In the setting of widespread necrosis, the contribution of non-parenchymal cells may be particularly important. Macrophages are essential in clearing toxic cellular debris[371], and thus mice deficient in CSF1 which promotes the maturation of macrophages have impaired liver regeneration[372], and CSF1 serum levels correlate with recovery from paracetamol liver injury[373].

Hepatic steatosis

Hepatic steatosis is known to be detrimental to liver regeneration not only in experimental models[374], but also in the clinical setting[375]. The mechanism is not fully understood but may include cell cycle machinery defects in steatosis[376]. Down regulation of the EGFR pathway may also contribute, and EGFR overexpression has been shown to rescue liver regeneration in a mouse model of PH in steatosis[377]. Steatosis may also compromise liver regeneration by inhibition of NFκB[378]. Finally, failure to activate growth arrest and DNA damage-inducible protein GADD34 in fatty liver may partly contribute to impaired liver regeneration, which can be rescued by transgenic overexpression of growth arrest and DNA damage-inducible protein GADD34 in a mouse experimental model[379].

Hepatic fibrosis and cirrhosis

In the setting of overwhelming injury where the capacity of hepatocytes to proliferate is overwhelmed, oval cell transdifferentiation provides a mechanism to assist cellular repopulation. Such injury is, however, also associated with activation of stellate cells to myofibroblasts, which secrete ECM[380]. Excessive ECM secretion is nonetheless harmful because it inhibits the ductular reaction[381] and hepatocyte proliferation [382]. Moreover, excessive fibrosis impairs portal flow, leading to arterialisation of the liver, and senescence of hepatocytes and cholangiocytes[383]. The distortion of the macro and micro-anatomy of the liver results in major compromise to liver function leaving affected patients in an extremely precarious state characterized by rapid and severe decompensation, which can be triggered by relatively minor physiologically stresses.

SECTION 7: MECHANISMS UNDERLYING CESSATION OF LIVER REGENERATION

In the rodent PH model, liver regeneration proceeds until the liver have returned to its pre-PH weight, approximately 10 d later, at which point regeneration ceases. The mechanisms resulting in termination of liver regeneration have received less attention than those driving it, but are equally important, not only from the perspective of the study of liver regeneration biology but also in terms of the light they shed on other pathologies including liver malignancy. Nevertheless, several regeneration termination pathways have been identified, relating to TGFβ, the activins, the ECM, and glypican-3 (GPC3).

TGF β

TGFβ is a multifunction cytokine with wide ranging roles in growth and development. It exists in 3 isoforms resulting from differential protein cleaving and binds to 3 different TGFβ receptors. Binding to the TGFβR results in autophosphorylation, and activation of SMAD, which translocates to the nucleus, delivering an inhibitory signal to cell proliferation[384]. Although TGFβ does inhibits hepatocyte proliferation *in vitro* [385], other experimental results *in vivo* cast some doubt on its role in termination of liver regeneration in that liver specific TGFβR knockout mice terminate liver regeneration appropriately[68]. However, this result does not necessarily rule out TGF β as a significant factor in liver regeneration termination: given the redundancy seen in the processes that drive regeneration, it seems likely that similar redundancy exists in its termination, such that ablation of one mechanism may readily be rescued by other pathways.

Activins

The activins are a family of proteins which are similar in structure to the TGFβ family, which also transduce signals *via* receptors that activate SMAD, and convey a growth inhibitory effect. Activins are upregulated during the liver regeneration[386], and blocking their action pharmacologically results in excessive regeneration and hepatomegaly following PH in rats[387].

ECM and integrin linked kinase

The ECM is thought to convey a growth controlling influence on liver cells in a mechanism whereby integrin proteins in intact ECM bind hepatocyte cell membrane Integrin linked kinase (ILK) receptors, which deliver a growth inhibitory signal[388]. Consistent with this, the growth response of hepatocytes to mitogens *in vitro* is much reduced when grown in the presence of ECM in comparison to plastic[389]. Moreover, ILK knockout mice show not only hepatomegaly in the native state[390], but also an exaggerated regeneration after PH[391]. Thus, it may be that the activation of matrix metalloproteases that occurs early after liver injury[188] results in degradation of the controlling influence of integrins, and that this is gradually recovered during regeneration as new ECM is laid down.

GPC3

GPC3 is a heparan sulphate proteoglycan found on the cell surface of many tissues which conveys a growth inhibitory effect[392]. It is not detectable in quiescent liver but is expressed coinciding with the end of regeneration after PH in rats[393]. Moreover, loss of function mutations of GPC3 results in organ overgrowth[394], and transgenic

over-expression of GPC3 delays liver regeneration after PH[393].

C/EBP

C/EBP is one member of a family of transcription factors with a role in producing cell cycle arrest. Although complete ablation of C/EBP is fatal in mice[395], altering the function of the protein by mutation results in partial loss of function with mice exhibiting excessive regeneration and hepatomegaly after PH and CCl4 injury[396], thus suggesting that the native protein has a role in the control of liver regeneration.

Cyclin E1 and E2

The cyclins are a group of proteins which impact on the progression of the cell through the cell cycle. Amongst these cyclins E1 and E2 influence the advancement of the cell from the G1 phase (during which the cell synthesizes protein and organelles), to S phase (during which DNA is replicated)[397]. Cyclin E1 and E2 have opposing roles, with Cyclin E1 promoting entry into S phase[398], and Cyclin E2 halting it[399]. Thus, mice with ablated Cyclin E2 show increased DNA synthesis and hepatomegaly after PH suggesting a role for Cyclin E2[398,400].

Of note the hepatomegaly seen after CyclinE2 ablation is not due to cell division, but hypertrophy, providing a possible mechanism to explain to the observation that liver growth after 30% PH is hypertrophic rather than the hyperplasia seen in 70% PH.

Hippo/YAP pathway

The *Hippo/Yap* signalling pathway is conserved in a wide range of organisms and associated with growth suppression[401]. *YAP* is key downstream effector of *Hippo* and its activation when dephosphorylated leads to massive liver overgrowth[402], suggesting a possible regulatory role in control of liver regeneration.

Micro RNAs

Micro RNAs are short RNA molecules which bind to messenger RNA and thus affect expression of the gene product by interfering with translation of mRNA to protein [403]. Several micro RNAs have been identified which target the mRNAs of key regeneration promoting proteins, and thus may play a part in controlling liver regeneration. Thus miR-23b targets the growth inhibiting SMAD protein such that the observed downregulation of miR-23b following PH may provide a mechanism for slowing liver regeneration[404]. miR-34a targets several mRNAs including that which codes for the HGF receptor MET, again providing a potential mechanism for limiting hepatocyte proliferation[405].

SECTION 8: LIVER REGENERATION: IMPLICATIONS FOR THERAPY OF LIVER TUMOURS

The complexity of liver regeneration biology, combined with our currently limited understanding has to date much restricted specific clinical interventions to enhance liver regeneration. Moreover, the processes involve such fundamental biochemical pathways that attempts to manipulate these would require very careful assessment, for fear of unintended consequences in the liver and other organ systems. Nevertheless, current knowledge allows clinicians to anticipate what scenarios or treatments may compromise liver regeneration and provides guiding principles which may allow planning treatment strategies to optimise liver regeneration potential.

Liver tumours, be they primary or metastatic, may be treated by chemotherapy in systemic or locoregional delivery methods, or by surgical intervention with resection or local ablation techniques. Radiotherapy, although used to an extent, has a much lesser role and evidence base. In this section, we discuss how the biology of liver regeneration affects treatment choices and delivery, not only in terms of chemotherapy and surgery, but also in relation to wider organ system physiology that relates to liver regeneration.

Chemotherapy and liver regeneration

The principal scenario in which chemotherapy impacts on liver regeneration is the use of neoadjuvant chemotherapy prior to a planned liver resection, or in the instance of downsizing an initially unresectable lesion, most usually in the context of colorectal liver metastases[406]. Chemotherapy affects not only individual cell types within liver parenchyma, but also key extra-hepatic tissues pertinent to the liver regenerative

process such as the bone marrow, and in some cases affects key liver regenerative pathways.

The main toxicities inflicted on the liver by chemotherapy are steatosis, steatohepatitis, and sinusoidal obstruction syndrome (SOS). Steatosis is the excessive deposition of fat within hepatocytes. This may trigger an inflammatory reaction leading to steatohepatitis, and in turn to fibrosis and cirrhosis. Sinusoidal obstruction syndrome is a separate entity with direct toxicity to LSEC leading to occlusive phenomena in the sinusoids[407].

In the context of treatment of (colorectal liver metastases) CRLM, 5FU is associated with steatosis[408], probably as a result of impaired oxidation of fatty acids[409]. 5FU also triggers the activation of pro-inflammatory genes, which may contribute to the evolution of steatohepatitis[410]. Chemotherapy associated Steatohepatitis (CASH) is also particularly associated with regimens containing irinotecan[411] which also inhibits fatty acid oxidation, but elicits steatohepatitis by activation of ERK[412], which if inhibited, leads to a reduction in steatohepatitis. SOS is associated with oxaliplatin regimens. Although not fully understood, a contributing mechanism is the increased expression of matrix metalloproteases, resulting in lifting of LSEC from the basement membrane and allowing infiltration of red blood cells into the space of Disse, thus causing an occlusive phenomenon in the lumen of the sinusoid, and an inflammatory reaction with stellate cell activation and perisinusoidal fibrosis[413].

In addition to these directly hepatotoxic effects, the above agents are myelosuppressive, and may thus compromise the bone marrow constituents that play important roles in liver regeneration, including (1) LSEC progenitors which are important in repopulating LSEC after PH and a key source of the important mitogen HGF[314,315]; (2) Macrophage progenitors which have a key role in clearing cell debris in preparation for regeneration[206]; and (3) Megakaryocytes which replenish platelets, with their important role in delivering liver regenerative signals[178,179].

5FU, oxaliplatin, and irinotecan are frequently used in combination with biological agents targeting specific proliferative pathways within tumours, which overlap with biochemical pathways promoting liver regeneration. Thus, the anti-EGFR antibody cetuximab blocks binding of EGFR ligands including the key mitogen EGF, acting *via* the RAS-RAF-MEK pathway. Another example is the anti-angiogenic antibody bevacizumab which delivers antitumor effects by blocking VEGF-binding to its receptor, but thereby also interfering with LSEC repopulation in the liver[406]. Thus, the commonly used chemotherapeutic agents and antibodies used in the treatment of CRLM have a multitude of liver regeneration compromising properties. The challenge for clinicians is to find the optimal balance between the oncological benefits and liver regenerative toxicity.

Clinical trials provide some guidance in this regard. Thus 5FU, oxaliplatin and irinotecan-based regimens have been shown to produce CRLM response rates allowing increased potential for curative resection[414,415], with additional benefits attributed to the use of anti EGFR[416] and anti VEGF antibodies[417]. Interestingly, whilst the use of EGFR antibody regimens is established as a means to downsize tumours to increase operability, their perioperative use in primarily resectable CRLM is detrimental[418].

In terms of duration of chemotherapy, the theoretical ideal is the delivery of the oncological hit to the tumour whilst minimising liver toxicity. In this regard Karoui *et al*[419] found that patient receiving fewer than 6 chemotherapy cycles experienced significantly fewer post liver resection complications than those who had received more than 6 cycles (19% *vs* 54% complication rate) although there was no impact on mortality rates. Similarly, Nguyen *et al* [420]showed a greater incidence of post-operative liver failure in patients undergoing more than 10 cycles of chemotherapy. Moreover, the general practice of leaving an interval of 4-6 wk between chemotherapy and surgery is intended to allow reversible inflammatory changes and bone marrow to recover[421].

The prospect of specific interventions to minimise chemotherapy related injury is the subject of research but has not yet reached widespread clinical application. Nevertheless, there have been reports that bevacizumab may reduce SOS in oxaliplatin regimens[422], and S-adenosylmethionine (SAMe) may have a protective effect in chemotherapy-induced liver injury[423], with SAMe infusion associated with lower serum concentrations of aspartate transaminase and alanine transaminase during chemotherapy treatment[424].

Liver volume manipulation

In the context of a healthy underlying liver parenchyma, up to 75% of the liver may be resected. However, this is the very limit and liver surgeons are often faced with the

problem of some degree of parenchymal pathology or dysfunction, which may present a higher requirement in terms of the remnant liver volume. As discussed in section 1.4, pure volume assessments are gradually being complemented by liver functional assessments, principally by hepatobiliary scintigraphy[146,152].

In instances where future remnant liver volume/function is deemed insufficient, PVE has become an established technique to produce atrophy of the tumour bearing liver parenchyma, and compensatory growth of the future remnant liver, allowing a safer hepatectomy[425]. In the context of resections for CRLM, this technique increases resection rates by 10%-20%[426].

In the instance where PVE fails to produce sufficient hypertrophy, perhaps caused by the development of collateral intrahepatic portal vessels between the embolized and non-embolized parts of the liver, further growth of the future remnant liver may be achieved by parenchymal section to interrupt the collateral vessels.

This approach, as a salvage manoeuvre after PVE is a modification of the originally described ALPPS technique[427] that combined single stage portal vein embolization and parenchymal transection. Whilst undoubtedly producing significant additional growth, the technique remains debated owing to questions regarding the functionality of the rapidly expanded liver, and high post-operative mortality in some series[428].

Liver disease limitations to liver resection

The continuum of steatosis, steatohepatitis, fibrosis, and cirrhosis presents a major challenge to liver resection. In terms of steatosis, the observed experimental and clinical compromise to liver regeneration as a result of steatosis translates to a diminished tolerance to liver resection[429]. Thus, studies examining the outcome of hepatic resection in patients with steatotic livers suggest more marked abnormalities in postoperative liver dysfunction, more morbidity and increased complication rates [143,430], with steatosis identified as an independent predictor of complications[431]. Increased mortality is identified in some studies[430], and although this has not been a universal finding, the presumption is that this relates to careful patient selection.

The tolerance of the liver to resection progressively decreases with more severe underlying liver disease. Thus, cirrhosis is associated with increased mortality rates for all abdominal surgery including[432] liver resection[433].

Interventions to mitigate the risk of liver surgery in the context of underlying liver disease are limited, and practice has focused on patient selection to avoid prohibitively hazardous resections. In the context of steatosis, the response of the steatotic liver to ischaemic insult[434] has motivated research in the concept of ischaemic preconditioning, whereby a short period of ischaemia prior to liver resection is applied with a view to improving subsequent perfusion[435]. Although benefits have been shown in rodent models and in the clinical setting[436], results have not been universal[437], and the practice not widely accepted.

The role of the gut

One of the key biochemical triggers which initiates liver regeneration is LPS from the gut, which translocates into portal blood following liver resection in part as a result of the rise in portal pressure[204] (see "*Priming of hepatocytes*"). The evidence supporting the importance of LPS is that germ free mice show impaired liver regeneration, which can be rescued by exogenous LPS administration[205].

Though the presence of LPS in the blood is important, there appears to be a delicate balance as excessive translocation of LPS into portal blood is detrimental to liver regeneration. Thus, in the rat model after 90% PH, gut mucosal permeability is disrupted with loss of tight junctions, resulting in high levels of portal blood LPS, severe inflammatory changes in the liver with necrosis, associated with high mortality. By decontaminating the gut with gentamycin, gut permeability, portal blood LPS, and liver necrosis is much improved, and associated with a significant improvement in survival from 24% to 56%[27].

No clinical trials have been carried out to test the potential benefit of this animal experimental result in humans, where its applicability could be investigated in patients undergoing major liver resection. Additional considerations would come into play, including the risk of *clostridium difficile* sepsis associated with alteration of bowel flora. Another potential approach, also uninvestigated, could be the use of bowel preparation similar to that used prior to colorectal surgery.

Multivisceral resections

The data from early experimental result suggested that liver regeneration was significantly dependent on portal blood growth factors derived from the upper

gastrointestinal tract (stomach, duodenum, pancreas) as portal flow separation experiments comparing isolated portal flow of distal stomach, duodenum, pancreas and spleen to portal flow of small intestine showed that the grafts supplied with intestinal portal flow atrophied, in contrast to those supplied with portal blood from the upper gastrointestinal tract[9,10].

On this basis, there might be a similar risk in humans in cases where liver resection is combined with a significant resection of the upper gastro-intestinal tract, such as Whipples resection (resection of distal stomach, duodenum and head of pancreas) or total pancreatectomy (resection of distal stomach, duodenum, pancreas, and spleen). However, existing case series describing such multivisceral resections do not report problems relating to liver regeneration[438-440].

Thus it may be that the situation in the original animal experiments cannot be extrapolated to humans owing to different physiology, although interpretation of the human literature has to be taken with the caveats of selective reporting (bias against reporting poor outcomes with liver failure), case selection of surgical candidates (likely good performance status individuals for such major resections), and likely small liver resections which might not manifest with post-operative liver failure.

Bile metabolism considerations

Bile salts are important auxiliary mitogens, with rodent models showing impaired liver regeneration with bile acid sequestering agents[243], bile-salt-deficient transgenic mice[244], and in rats with external biliary fistula[245].

This observation in rodent models is mirrored in clinical practice, with a randomised trial comparing patients undergoing major liver resection with and without cystic duct biliary drainage showing significantly lower bile salt concentrations in the drained group as well as lesser liver regeneration assessed by volumetric CT[441]. Moreover, in the context of portal vein embolisation, increased systemic bile salt levels predicted hypertrophy of the non-embolised lobe[442].

Thus, the presence of bile in the intestine following liver resection appears to be important and would argue against the use of external biliary drains after liver resection, or in circumstances requiring such drains, to consider enteric bile recycling *via* nasogastric or nasoenteric tube.

Management of post-hepatectomy portal hypertension

The rise in portal venous pressure following liver resection appears to contribute to providing important triggers to liver regeneration in the form of induction of cytokines, hepatic mitogens and angiogenic growth factors, as described in "*Vascular events*"section. However, an excessive increase in portal pressure is thought to be detrimental in eliciting a reduced arterial inflow as a result of the arterial buffer response, and a subsequent hypoxia[171], which is hyptothesised to contribute to post liver resection liver failure[443], in combination with direct mechanical injury occurs to the liver sinusoids[444].

Thus, a number of investigators have examined a variety of interventions to decrease portal venous pressure by surgical (splenectomy, splenic artery ligation, porto-systemic shunt), interventional radiological (pre-operative splenic artery ligation), and pharmacological means (non-selective beta-blockers, terlipressin), with successful reductions in portal pressure and improvement in small-for-size syndrome [444].

Hypoxia

Intrahepatic hypoxia is one of the stimuli which may contribute to the early triggers of liver regeneration[175], *via* a number of mechanisms including the hypoxia induced secretion of complex regeneration promoting molecules from stem cells[176]. Given the critical necessity of maintaining normoxia in other tissues, manipulating pO2 for hepatic regeneration benefits seems an unlikely strategy, however, pharmacological manipulation of HIF has been used to treat renal anaemia[445], and *in vitro* studies suggest that such agents could produce angiogenesis in the liver, as well as a hepatocyte cytoprotective effect[174].

Adrenergic stimulation

The finding that surgical denervation of the liver or pharmacological alpha adrenergic blockade significantly impairs liver regeneration, is consistent with known modulatory effects of catecholamines on secretion of hepatic mitogens (including EGF), as well as the finding that catecholamines increase hepatocyte sensitivity to EGF[251]. Moreover, hepatic stellate cells and hepatic progenitor cells are innervated by the

sympathetic and parasympathetic nervous system[446].

Hepatic sympathectomy is effectively carried out in humans in the context of hilar cholangiocarcinoma resections (when the entire hepatic hilum is skeletonized) and in liver transplantation, when the implanted graft is totally denervated, however there is no obvious clinical evidence that this has a detrimental effect on liver regeneration.

The absence of reported clinical compromise to liver regeneration following sympathectomy in humans may reflect differences in autonomic supply[447], compensatory effects from adrenal catecholamine secretion, or perhaps relatively rapid re-innervation, as seen in some animal models[448]. Finally, the effect of catecholamine stimulation is complicated by the fact that inhibition of alpha adrenergic signals may have a beneficial effect in minimising hepatic stellate cell activation and therefore reduce detrimental excessive fibrosis[446].

The role of platelets and fibrinogen

Platelets clearly have an important role in promoting liver regeneration with animals and humans studies showing impaired liver regeneration in individuals thrombocytopenia[178,179], and the association between thrombocytopenia and post-operative mortality after major liver resection[449]. Moreover, fibrinogen deposition in the liver appears key in driving platelet accumulation in the liver, with post hepatectomy hypofibrinogenaemia being associated with liver dysfunction and mortality. Moreover, studies in rodents suggest platelets are the key source of the auxiliary hepatic mitogen serotonin in liver regeneration, and that serotonin infusion rescues impaired liver regeneration observed in thrombocytopenic mice[256].

Correction of platelet count and fibrinogen levels in patients post hepatectomy has not been investigated in humans, but could potentially have beneficial effects, although thromboembolic risks would have to be taken into account. Serotonin infusion has also not been investigated in humans in the context of liver regeneration, but has been carried out in other clinical contexts to stimulate prolactin[450], and as a desired effect in amitriptyline treatment of depression[451].

Non parenchymal cell modulation

In the setting of liver regeneration with significant hepatic necrosis, the role of non-parenchymal cells may be particularly important. Macrophages clear toxic cellular debris, and mice deficient in macrophage colony stimulating factor 1 (CSF1) which promotes the maturation of macrophages have impaired liver regeneration[372]. Moreover, CSF1 correlates with recovery from paracetamol liver injury in patients with liver failure[373]. In addition to clearing metabolic debris, macrophages may in part act by stimulating the hepatic ductular reaction[452] and limiting fibrosis[453].

Although macrophage colony-stimulating factor-1 has not been used in humans in the context of liver failure and regeneration, it does have a multitude of other clinical applications[454].

Similarly, in rodent models at least, the bone marrow is a significant source of LSEC progenitor cells, which emigrate from the marrow into the bloodstream from which they engraft into regenerating liver, where they produce a significant quantities of the complete hepatic mitogen HGF, in a VEGF driven process[314,315]. Specific LSEC progenitor stimulation therefore offers a theoretical therapeutic opportunity but has not been investigated.

Modifying the proliferative response.

The characterization of the multiple cytokines, hormones and growth factors involved in liver regeneration has motivated investigation of means to modulate the hepatic proliferative response. Such approaches have involved the infusion of specific mitogens, or the use growth enhancing progenitor cells or their secretome and are to date at experimental animal model stage.

These wide-ranging experimental approaches are beyond the scope of this review, but a few examples provide an insight into potential avenues for the future. In a murine model of 85% PH, Cataldegirmen *et al*[455] investigated the potential therapeutic opportunity of blocking the Receptor for advanced glycation end-products (RAGE), which is upregulated in massive hepatectomy and associated with cell stress when binding its ligands. Blockage of RAGE pharmacologically or by transgenic means resulted in significant improvement in survival post massive PH[455]. In a mouse model of partial biliary ligation, Mangieri *et al*[456] report improved liver regeneration produced by infusion of the complete mitogen HGF. Similarly, but targeting the other complete mitogen pathway of the EGFR ligands, Zimmers *et al*[377] demonstrated improved liver regeneration after plasmid delivery of EGF receptor.

In addition to the focus on individual hepatic mitogens, investigation has also been carried out in the infusion of whole cells[457], ranging from primary human hepatocytes[458], pluripotent stem cell derived hepatocyte-like cells[459], and mesenchymal stem cells[460]. Cell based therapies have met several obstacles including sourcing, immuno-compatibility, and potential malignant transformation, thus motivating research into potential for using the secretome of stem cells, thus obviating the difficulties presented by the whole cell therapies[176,461-463].

CONCLUSION

Liver regeneration is highly complex, and current understanding is based largely on animal and *in vitro* models. The likelihood that not all hepatic mitogens have been identified, the multitude of known ones, the complexity and incomplete understanding of their associated biochemical pathways, the equally complex and poorly understood cross talk between cell types, and our even poorer understanding of the factors that cease liver regeneration all suggest that a comprehensive working understanding of the process is improbable in the foreseeable future. Consequently, specific interventions to influence liver regeneration in the clinical setting are commensurately limited, though allow clinicians to at least optimise conditions for liver regeneration to occur. The implications of this in relation to the treatment of liver tumours are most notably applicable in the context of liver resection for malignancy, where assessment and optimisation of remnant liver function not only increases the proportion of patients eligible for treatment, but also improves patient safety. The increasingly sophisticated *in vitro* organoid models, and potential opportunities presented by repopulation of decellularised scaffolds may allow the creation of constructs that allow not only deeper understanding, but also novel therapeutic options.

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REVIEW

Primary vascular tumours of the kidney

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Abstract

Primary vascular tumours of the kidney are rare and may pose diagnostic difficulties because of their similar clinical, morphological, and immunohistochemical features. This article summarizes the clinical and pathological features of primary renal angiosarcoma and anastomosing haemangioma of the kidney including epidemiology, genetics, and prognosis. Renal anastomosing haemangiomas are benign neoplasms characterized by anastomosing capillary-sized vascular channels. These tumours are rare, with about 75 cases reported in the literature. Most anastomosing haemangiomas are found incidentally on ultrasound, computed tomography, or magnetic resonance imaging. Common symptoms include abdominal pain, haematuria, and abdominal mass. Renal anastomosing haemangiomas are characterized by recurrent mutations in *GNAQ* and *GNA14* genes. The prognosis of anastomosing haemangioma is excellent.

Primary renal angiosarcomas are malignant tumours showing endothelial differentiation. To date, 76 cases have been described in the literature. Primary renal angiosarcomas are frequently symptomatic. The clinical features of renal angiosarcomas are similar to those of renal anastomosing haemangiomas, including abdominal pain, haematuria, and abdominal mass. Angiogenesis-related genes and vascular-specific receptor tyrosine kinases such as KDR, TIE1, SNRK, TEK, and FLT1 are upregulated in angiosarcomas. Primary renal angiosarcomas are highly aggressive neoplasms with a poor prognosis despite surgical treatment, chemotherapy, radiotherapy, or targeted therapy.

Key Words: Kidney; Renal tumours; Angiosarcoma; Haemangioma; Anastomosing haemangioma of the kidney; Vascular tumours

Core Tip: Primary vascular tumours of the kidney are extremely rare. This article

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INTRODUCTION

Although vascular tumours are relatively common in the skin and soft tissue, they are extremely rare in the kidney, ranging from benign to malignant neoplasms that may be diagnostically challenging because of the overlapping clinical, morphological and immunohistochemical features.

These tumours include renal angiosarcomas and renal haemangiomas. Various subtypes of haemangioma have been described in the kidney including cavernous, capillary, and anastomosing haemangiomas[1-4]. However, the most common subtype is anastomosing haemangioma[1,2,5].

This article provides an overview of the clinical and pathological features of anastomosing haemangioma of the kidney and primary angiosarcoma of the kidney, and discusses the epidemiology, genetics, and prognosis.

ANASTOMOSING HAEMANGIOMA OF THE KIDNEY

Renal anastomosing haemangiomas are benign neoplasms characterized by anastomosing capillary-sized vascular channels. These tumours are exceptionally rare with about 75 anastomosing haemangiomas reported in the literature[5-9]. These tumours occur in a wide age range from 10 to 83 years (mean, 49 years) with a male-to-female ratio of 2:1[10].

The aetiology and risk factors for renal anastomosing haemangiomas are unknown. Some cases have been reported in the setting of end stage renal disease[11,12].

The vast majority of anastomosing haemangiomas are found incidentally on radiological evaluation for other purposes. Common symptoms include abdominal pain, haematuria, and abdominal mass[5,10].

The imaging findings are non-specific. On computed tomography, these tumours are often circumscribed, hyperdense, and heterogeneous due to fatty or non-enhancing hypodense areas and show post-contrast enhancement[13].

Renal anastomosing haemangiomas are characterized by recurrent mutations in *GNAQ* and *GNA14* genes[14,15]. *GNAQ* gene encodes guanine nucleotide-binding protein G (q) subunit alpha (Gαq protein) that activates signalling pathways that regulates cell proliferation, survival, development, and function of blood vessels[14-16].

Grossly, anastomosing haemangiomas are typically small ranging from 0.1 cm to 12 cm (mean, 2.2 cm)[5,10,17]. These tumours are often well-demarcated spongy mahogany brown masses[5]. They are usually unilateral and solitary tumours; however, a few cases of bilateral[18] and multifocal[11] tumours have been described.

Histologically, anastomosing haemangiomas consist of anastomosing capillary-sized blood vessels, reminiscent of splenic sinusoids. The blood vessels are lined by bland endothelial cells. Typically, these tumours lack endothelial cell multilayering, papillary tufting, cytologic atypia, necrosis, and prominent mitotic figures. They may show extramedullary haematopoiesis, hyaline globules, and mild lymphocytic infiltrate[1,2,5,10,18].

Rarely, renal anastomosing haemangiomas may infiltrate perinephric fat, renal sinus fat[2,12], and the renal vein or its segmental branches[2,12,18,19]. The neoplastic cells are immunoreactive for CD31, CD34, ERG, FLI1, and factor VIII-related antigen (now rarely used)[5].

Renal anastomosing haemangiomas may co-exist with other renal neoplasms such as metanephric adenoma, papillary adenoma, papillary renal cell carcinoma, acquired cystic disease-associated renal cell carcinoma, and clear cell renal cell carcinoma[11,12].

Most patients with renal anastomosing haemangioma, described in the literature, were treated with radical nephrectomy, reflecting a tendency to overtreat these patients, probably because of inaccurate preoperative diagnosis (Table 1).

The prognosis of anastomosing haemangioma is excellent with no evidence of recurrence, metastasis or tumour-related death at an average follow-up of 24.8 mo (range, < 1-156 mo) (Table 1).

PRIMARY ANGIOSARCOMA OF THE KIDNEY

Renal angiosarcomas are malignant tumours showing endothelial differentiation. These tumours are very rare, with about 76 cases described in the literature as case series and reports[20-24]. There is a male-to-female ratio of 6:1, with patient age ranging from 24 years to 95 years (median, 62 years).

Although angiosarcomas arising in other anatomical sites have been associated with risk factors such as exposure to thorium dioxide, arsenic-based pesticides, polyvinyl chloride, and radiation therapy particularly for breast, endometrial and prostate cancers[25-27], no specific aetiology or risk factors have been established for primary angiosarcoma of the kidney[20,28].

The clinical features of renal angiosarcomas are identical to those of renal anastomosing haemangiomas. Common symptoms include abdominal pain, haematuria, abdominal mass, and weight loss. A rare case of spontaneous tumour rupture with retroperitoneal haematoma has been described[29].

Computed tomography imaging shows large masses with heterogeneous enhancement and hypervascularity[30].

Angiogenesis-related genes and vascular-specific receptor tyrosine kinases such as KDR, TIE1, SNRK, TEK, and FLT1, are upregulated in angiosarcomas[31]. High-level MYC gene amplifications are seen in most radiation-induced and chronic lymphoedema-associated angiosarcomas[32]. A subset of cases is characterized by PLCG1, KDR, and PTPRB mutations[33,34].

Some primary angiosarcomas, typically in young adults, have recurrent CIC gene rearrangements, with or without concurrent CIC mutations, and are characterized by upregulation of CIC-target genes including *ETV1*, *ETV4*, and *ETV5*[34]. Angiosarcomas with CIC gene abnormalities are associated with an inferior disease-free survival[34].

Primary renal angiosarcomas are usually large ranging from 3.6 cm to 30 cm (mean, 13 cm). Typically, they are ill-defined haemorrhagic spongy masses with necrosis[1,20].

Microscopically, these tumours range from well-formed vasoformative areas to areas with solid morphology showing sparse vasoformation. These patterns are often mixed within the same tumour. Vasoformative areas are composed of small to medium-sized anastomosing blood vessels, lined by epithelioid and/or spindled endothelial cells showing nuclear pleomorphism, endothelial papillary tufting, multilayering, intraluminal budding, and hobnailing[1,20,28]. Solid areas consist of sheets of malignant epithelioid and/or spindled cells with subtle vasoformation, cytologic atypia, and mitotic figures. Necrosis may be present. Angiosarcomas usually have a haemorrhagic background and extravasated red blood cells are seen within the tumour[1,20,28].

Epithelioid angiosarcomas are composed of sheets of large atypical polygonal or epithelioid cells with nuclear pleomorphism, high nuclear to cytoplasmic ratio, prominent central nucleoli, mitotic figures, and moderate amounts of cytoplasm. Epithelioid angiosarcomas may be mistaken for carcinoma, melanoma, or lymphoma [20,28,35-37].

The neoplastic cells are positive for CD31, ERG, FLI1, CD34, and factor VIII-related antigen[20,28,38,39]. Epithelioid angiosarcomas may be positive for epithelial markers including CK7, Cam5.2, AE1/AE3, and EMA, which may lead to a misdiagnosis of carcinoma[20].

Similar to angiosarcomas arising at other locations, renal angiosarcomas have a tendency for widespread metastasis at diagnosis or afterwards in the course of the disease. Approximately 66% of patients develop metastases, most commonly to the lung and liver. Other sites of metastasis include bone, lymph nodes, peritoneum, small bowel, soft tissue, and skin. Currently, there are no specific standardized treatment guidelines for primary renal angiosarcomas. These tumours are treated with radical nephrectomy, chemotherapy, radiotherapy, or targeted therapy (Table 2).

Table 1 Treatment, follow-up, and outcome of patients with anastomosing haemangioma of the kidney

Ref.	Treatment	Follow-up (mo)	Outcome
Bean *et al*[14]	Nephrectomy	9	NED
Bean *et al*[14]	Nephrectomy	84	NED
Bean *et al*[14]	Nephrectomy	107	NED
Memmedoğlu and Musayev[41]	Nephrectomy	12	NED
Memmedoğlu and Musayev[41]	Nephrectomy	12	NED
Tahir and Folwell[42]	Nephrectomy	1	NED
Pantelides *et al*[6]	Nephrectomy	6	NED
Downes *et al*[7]	Nephrectomy	NA	NA
Downes *et al*[7]	Biopsy	NA	NA
Chandran *et al*[8]	Nephrectomy	NA	NA
Cha *et al*[9]	Nephrectomy	5	NED
Montgomery and Epstein[2]	Nephrectomy	12	NED
Montgomery and Epstein[2]	Nephrectomy	36	NED
Montgomery and Epstein[2]	Nephrectomy	NA	NA
Montgomery and Epstein[2]	Excision	8	NED
Heidegger *et al*[43]	Nephrectomy	156	NED
Kryvenko *et al*[12]	Nephrectomy	NA	NA
Kryvenko *et al*[12]	Nephrectomy	NA	NA
Kryvenko *et al*[12]	Nephrectomy	NA	NA
Kryvenko *et al*[12]	Nephrectomy	NA	NA
Kryvenko *et al*[12]	Nephrectomy	NA	NA
Kryvenko *et al*[12]	Nephrectomy	NA	NA
Kryvenko *et al*[12]	Nephrectomy	NA	NA
Kryvenko *et al*[12]	Nephrectomy	NA	NA
Kryvenko *et al*[12]	Nephrectomy	NA	NA
Kryvenko *et al*[12]	Nephrectomy	NA	NA
Kryvenko *et al*[12]	Nephrectomy	NA	NA
Kryvenko *et al*[12]	Nephrectomy	NA	NA
Al-Maghrabi and Al-Rashed[44]	Partial nephrectomy	12	NED
Caballes *et al*[17]	Nephrectomy	18	NED
Büttner *et al*[11]	Nephrectomy	NA	NA
Büttner *et al*[11]	Nephrectomy	NA	NA
Büttner *et al*[11]	Nephrectomy	NA	NA
Büttner *et al*[11]	Nephrectomy	NA	NA
Büttner *et al*[11]	Nephrectomy	NA	NA
Büttner *et al*[11]	Nephrectomy	NA	NA
Büttner *et al*[11]	Nephrectomy	NA	NA
Büttner *et al*[11]	Nephrectomy	NA	NA
Lee *et al*[45]	Nephrectomy	NA	NA
Zhao *et al*[46]	Nephrectomy	12	NED
Kryvenko *et al*[18]	Nephrectomy	7	NED

Kryvenko *et al*[18]	Nephrectomy	6	NED
Kryvenko *et al*[18]	Nephrectomy	3	NED
Kryvenko *et al*[18]	Nephrectomy	122	NED
Tao *et al*[47]	Nephrectomy	21	NED
Abboudi *et al*[48]	Nephrectomy	<1	NED
Silva *et al*[49]	Resection	NA	NA
Berker *et al*[50]	Partial nephrectomy	10	NED
Berker *et al*[50]	Nephrectomy	4	NED
O'Neill *et al*[13]	NA	NA	NA
O'Neill *et al*[13]	NA	NA	NA
O'Neill *et al*[13]	NA	NA	NA
O'Neill *et al*[13]	NA	NA	NA
O'Neill *et al*[13]	NA	NA	NA
O'Neill *et al*[13]	NA	NA	NA
O'Neill *et al*[13]	NA	NA	NA
Brown *et al*[1]	Nephrectomy	72	NED
Brown *et al*[1]	Nephrectomy	24	NED
Brown *et al*[1]	Partial nephrectomy	NA	NA
Brown *et al*[1]	Nephrectomy	24	NED
Brown *et al*[1]	Nephrectomy	NA	NA
Perdiki *et al*[51]	Partial nephrectomy	25	NED
Perdiki *et al*[51]	Nephrectomy	14	NED
Wetherell *et al*[52]	Nephrectomy	1	DFUD
Manohar *et al*[53]	Nephrectomy	24	NED
Omiyale *et al*[19]	Nephrectomy	10	NED
Johnstone *et al*[54]	Nephrectomy	NA	NA
Mehta *et al*[4]	Nephrectomy	3	NED
Mehta *et al*[4]	Nephrectomy	12	NED
Mehta *et al*[4]	Nephrectomy	3	NED
Tran and Pernicone[55]	Nephrectomy	NA	NA
Zhang *et al*[56]	Partial nephrectomy	16	NED
Cheon *et al*[57]	Nephrectomy	6	NED
Chou *et al*[58]	Nephrectomy	8	NED
Chou *et al*[58]	Nephrectomy	14	NED

DFUD: Died from unrelated disease; NED: No evidence of disease; NA: Not available.

Primary renal angiosarcomas are highly aggressive neoplasms with 76.3% of patients dying of tumour within 1 mo to 24 mo (mean, 7.5 mo), despite surgical and adjuvant therapy (Table 2). Poor prognostic factors for angiosarcomas occurring at other anatomic sites include age > 69 years, tumour size ≥ 5 cm, regional disease (*vs* localized disease), non-surgical treatment, and distant metastasis[40].

CONCLUSION

Primary vascular tumours of the kidney are rare neoplasms. Unlike primary renal

Table 2 Treatment, follow-up, and outcome of patients with primary angiosarcoma of the kidney

Ref.	Treatment	Follow-up (mo)	Outcome
Costero-Barrios *et al*[59]	Nephrectomy, Chemo, RT	12	AWD
Peters *et al*[60]	Nephrectomy	2	DOD
Singh *et al*[35]	NA	NA	NA
Kern *et al*[61]	Nephrectomy	3	DOD
Kern *et al*[61]	Nephrectomy	1.5	DOD
Aydogdu *et al*[62]	Nephrectomy	NA	NA
Akkad *et al*[63]	Nephrectomy	30	NED
Witczak *et al*[64]	nephrectomy	NA	NA
Chaabouni *et al*[38]	Nephrectomy	1	DOD
Johnson *et al*[65]	Rapid deterioration	NA	DOD
Zenico *et al*[66]	Nephrectomy	4	DOD
Nguyen *et al*[67]	Nephrectomy, Chemo	18	DOD
Terris *et al*[68]	Nephrectomy, RT	10	DOD
Matter *et al*[69]	Nephrectomy, Chemo, RT	18	DOD
Yoshida *et al*[70]	Nephrectomy, Recombinant IL-2	13	DOD
Pauli and Strutton[71]	Nephrectomy, RT	2	DOD
Martínez-Piñeiro *et al*[72]	Nephrectomy, S	4	DOD
Bernstein *et al*[73]	NA	NA	NA
Liu *et al*[36]	Nephrectomy, RT	6	NED
Yau *et al*[74]	Nephrectomy, Chemo, RT	3	DOD
Carnero López *et al*[75]	Nephrectomy, Chemo	5	DOD
Kazaz *et al*[76]	Nephrectomy, Chemo	NA	NA
Souza *et al*[77]	Nephrectomy	1	DFUD
Detorakis *et al*[78]	Nephrectomy, Chemo	11	DOD
Komoto *et al*[79]	Nephrectomy	9.2	DOD
Boni *et al*[80]	Nephrectomy, Chemo	15	DOD
Chang *et al*[81]	Nephrectomy, Chemo, RT	NA	NA
Iannaci *et al*[82]	Nephrectomy	NA	DOD
Subramanian *et al*[83]	Nephrectomy	NA	NA
Waqas *et al*[84]	Nephrectomy, Chemo	NA	NA
Gourley *et al*[85]	Nephrectomy	NA	DOD
Su[86]	Nephrectomy, Chemo	NA	DOD
López Cubillana *et al*[87]	Nephrectomy, Chemo	5	DOD
Juan *et al*[88]	Nephrectomy, Chemo, RT	9	DOD
Prince[21]	Nephrectomy, RT	NA	A and W
Sesar *et al*[22]	Nephroureterectomy	NA	NA
Testa *et al*[23]	Nephrectomy	27	DFUD
Xuan[24]	Nephrectomy	NA	NA
Brown *et al*[1]	NA	6	DOD
Brown *et al*[1]	NA	11	DOD
Brown *et al*[1]	NA	1	DOD

Brown *et al*[1]	NA	NA	NA
Brown *et al*[1]	NA	1	DOD
Brown *et al*[1]	Nephrectomy	NA	NA
Brown *et al*[1]	Nephrectomy	NA	NA
Brown *et al*[1]	Nephrectomy	2	DFUD
Hiratsuka *et al*[89]	Nephrectomy	29	NED
Adjiman *et al*[90]	Nephrectomy	NA	DOD
Limmer *et al*[91]	Nephrectomy	1	DOD
Darlington *et al*[92]	Nephrectomy, Chemo	12	NED
Allred *et al*[93]	Nephrectomy, Chemo	3	DOD
Fukunaga *et al*[94]	Nephrectomy	13	DOD
Desai *et al*[95]	Nephrectomy, Chemo	4	DOD
Sabharwal *et al*[96]	Nephrectomy, Chemo	>1	NA
Aksoy *et al*[29]	Nephrectomy, S	3	DOD
Heo *et al*[97]	Nephrectomy	NA	NA
Mordkin *et al*[98]	Nephrectomy, Chemo, S	NA	NA
Berretta *et al*[99]	Nephrectomy, Chemo	8	DOD
Lodhi *et al*[100]	Chemo	NA	AWD
Cason *et al*[101]	Nephrectomy, RT	10	DOD
Askari *et al*[102]	Nephrectomy	4	DOD
Guan *et al*[103]	Nephrectomy, Chemo	4	DOD
Papadimitriou *et al*[104]	Nephrectomy	NA	A and W
Celebi *et al*[105]	Nephrectomy, Chemo, TKI, VEGF Inhibitor	13	DOD
Rüb *et al*[106]	Nephrectomy, Chemo	12	AWD
Zhang *et al*[107]	Nephrectomy	NA	NA
Tsuda *et al*[108]	Nephrectomy	21	DOD
Grapsa *et al*[109]	NA	NA	NA
Li *et al*[37]	NA	NA	NA
Qayyum *et al*[110]	Palliative (patient's decision)	NA	NA
Leggio *et al*[30]	Nephrectomy	8	DOD
Garmendia *et al*[111]	Nephrectomy	NA	NA
Sanyal *et al*[112]	Nephrectomy, RT	24	DOD
Cerilli *et al*[113]	Nephrectomy, RT	6	DOD
Douard *et al*[114]	Nephrectomy	3	DOD
Yamamoto *et al*[115]	Nephrectomy, RT	19	NED

RT: Radiotherapy; Chemo: Chemotherapy; TKI: Tyrosine kinase inhibitor; S: Splenectomy; DOD: Died of disease; AWD: Alive with disease; A and W: Alive and well; DFUD: Died from unrelated disease; NED: No evidence of disease; NA: Not available.

angiosarcoma, the prognosis of renal anastomosing haemangioma is excellent with no evidence of recurrence or metastasis. These tumours share similar clinical, morphological and immunohistochemical features, and must be distinguished from each other. Features that favour angiosarcomas include the presence of malignant spindled and/or epithelioid cells with a variable degree of vasoformation, cytologic atypia, prominent mitotic figures, endothelial multilayering, papillary tufting, and necrosis.

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MINIREVIEWS

Detection of circulating tumour cells in colorectal cancer: Emerging techniques and clinical implications

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Abstract

Despite several advances in oncological management of colorectal cancer, morbidity and mortality are still high and devastating. The diagnostic evaluation by endoscopy is cumbersome, which is uncomfortable to many. Because of the intra- and inter-tumour heterogeneity and changing tumour dynamics, which is continuous in nature, the diagnostic biopsy and assessment of the pathological sample are difficult and also not adequate. Late manifestation of the disease and delayed diagnosis may lead to relapse or metastases. One of the keys to improving the outcome is early detection of cancer, ease of technology to detect with uniformity, and its therapeutic implications, which are yet to come. "Liquid biopsy" is currently the most recent area of interest in oncology, which may provide important tools regarding the characterization of the primary tumour and its metastasis as cancer cells shed into the bloodstream even at the early stages of the disease. By using this approach, clinicians may be able to find out information about the tumour at a given time. Any of the following three types of sampling of biological material can be used in the "liquid biopsy". These are circulating tumour cells (CTCs), circulating tumour DNA, and exosomes. The most commonly studied amongst the three is CTCs. CTCs with their different applications and prognostic value has been found useful in colorectal cancer detection and therapeutics. In this review, we will discuss various markers for CTCs, the core tools/techniques for detection, and also important findings of clinical studies in colorectal cancer and its clinical implications.

Key Words: Circulating tumour cells; Colorectal cancer; Tools and techniques; Clinical implications

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Core Tip: Circulating tumour cells (CTCs) in the blood have been found to be mainly associated with the stage of the disease and serve as a prognostic marker for survival in colorectal cancer. Some studies have also reported its role in the diagnosis and treatment monitoring. By focusing molecular research on rare CTCs, targeting cellular markers of CTCs, and discovering new cellular markers may improve the management of colorectal cancer and play a role in prevention of metastatic disease. Patients at high risk might benefit from additional individualized treatment which can be investigated in future clinical trials.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers, which stands second and third in women and men, respectively, across the globe with more than 1.2 × 10^6 new cases and 608700 mortalities annually[1]. It develops due to genetic and epigenetic alterations in human genome and environmental factors. Mode of presentation of CRC can be inherited, familial, and sporadic. Inherited CRC accounts for 5%-10% of all cases, for example, Lynch syndrome, familial adenomatous polyposis, and Peutz-Jeghers syndrome. Among all the CRCs, familial CRC accounts for 20%-30% and sporadic cases approximately 70% of all CRCs which are associated with somatic mutations[2]. There are many invasive and non-invasive diagnostic and prognostic tools with varying sensitivity and specificity, and each has its limitation. There is a need for new tools which may be simpler, non-invasive, cheaper, reproducible, and easily available with high sensitivity and specificity. "Liquid biopsy" is currently the most recent area of interest in oncology, which may provide important tools regarding the characterization of the primary tumour as well as metastasis because tumour cells shed into the bloodstream at the early stages of the disease. In "Liquid biopsy", one of the three types of sampling of biological material can be used, which are-circulating tumour cells (CTCs), circulating tumour DNA, and exosomes. CTCs are one of the main components of liquid biopsy, where subsets of tumour cells can disseminate from the primary tumour and intravasate to the circulatory system. CTCs are non-invasive and safe in comparison to traditional tissue biopsy, and can be used for monitoring of tumour progression and tumour response to therapy in real time. CTCs in peripheral blood serve as a source of valuable tumour markers. The present review will describe the main areas of the ongoing investigation on CTCs with particular emphasis on different tools and techniques used for CTC capturing and analysis, and also currently available data of clinical relevance of CTCs.

CTCs

A tumour cell contains millions of cells maintaining genetic mutations driving them to grow, divide, and invade the local tissues. Some cells separate from the edges of a tumour and are released into the bloodstream or lymphatic system. These cells are CTCs. CTCs can also be defined as cells spreading into vasculature by a primary tumour and they keep circulating in the bloodstream of cancer patients[3]. It was Ashworth (1869) who reported the CTCs for the first time and described the presence of tumour cells with resemblance to the cells from the primary tumour, in the blood of a patient with metastatic breast carcinoma. Later, in 1955, evidence of the presence of CTCs in the blood of a patient with primary and metastatic carcinoma was found by immunohistochemistry. In 1990, Moss and Sanders in their study found evidence for CTCs in seven out of ten disseminated neuroblastoma patients by immunostaining. In CRC, CTCs were first reported in 1993 with the help of conventional cytology and cytokeratin staining. Tumour cells were isolated from 42 patients who underwent

resection with the help of density gradient centrifugation, immune histological evidence for CTCs was reported in 4 out of 42 patients. Above mentioned studies have showed that tumour cells could be detected by traditional immunochemistry techniques; however, their results were based on small sample size and single-center studies.

Some studies have also reported CTC circulation in the body fluids before metastasizing to other parts of the body even in the early stages of the disease[4,5]. Wang *et al*[6] analysed the prognostic role of CTCs, highlighting the importance of CTC count before and after chemotherapy. They found that the presence of CTCs during chemotherapy is an unfavorable but independent factor and may play a role in deciding overall survival (OS) and survival without disease progression [progression free survival (PFS)] in advanced CRC cases. From this study, it was clear that CTCs in peripheral blood can be used as useful tumour markers. Characterization and early detection of CTCs have been reported to play an important role as a prognostic and predictive factor in different types of solid tumours[7,8]. Many epithelial cancers, including breast, prostate and lung cancers, have also been found to be associated with CTCs[9,10].

Early diagnosis, prediction of prognosis, assessment of recurrent risk, individualized treatment, and treatment with curative intent have focused research in the field of CTCs[11]. CTCs have faced difficulties for years because of their very low number (1–10 cells *per* 10 mL of blood) in many studies, and they have a short half-life which ranges from 1 to 2.4 h in blood[12,13], hence posing difficulty in further study. Their detection, quantification, and characterization of molecular features are also difficult. At present, there are several limitations to available CTC isolation techniques. Moreover, only a very small number of CTCs possess metastatic property[14]. Hence, it is very important to characterize them exactly so as to differentiate the non-metastatic CTCs from metastatic ones. There are several techniques which are described here for isolation and detection of CTCs effectively.

CIRCULATING TUMOUR CELL ISOLATION AND DETECTION METHODS

Basic principles

Investigation of CTCs can provide helpful clinical information. However, as described earlier, blood stream harbors very few CTCs and every single CTC is surrounded by 10^6-10^7 mononuclear white blood cells (WBCs). To isolate CTCs and detect their characteristics, it is crucial to isolate them from whole blood cells.

Although there are several methods described for isolation of CTCs (Table 1), there are only two basic approaches. The first one is isolation methods based on the detection of specific surface markers for CTCs, which is also termed as "label-dependent methods" (or cell surface markers), and the second method is based on physical or biological properties of CTCs, termed as "label-independent methods". These approaches are not based on antibodies or other markers for labeling the cells of interest, but they enrich them by use of the difference of physical properties.

Label-dependent methods

In these methods, CTC isolation is based on specific markers. The majority of label-dependent methods use specific epithelial tissue marker-epithelial cell adhesion molecule (EpCAM). EpCAM is the most commonly used method of capturing CTCs because its expression is virtually universal in the cells of epithelial origin and is absent in blood cells. Cell capture with conjugated antibodies followed by purification of captured cells *via* the magnetic field was initially used to enrich CTCs from the blood of patients with prostate or breast cancer. The CellSearch system (Veridex) is a commercial platform which is based on this feature; CTCs are characterized as a population of EpCAM-captured cells that are confirmed to be negative for CD45 and positive for cytokeratins[15]. Other markers are also used, like human epidermal growth factor receptor 2 (HER2), mucin 1 (MUC1), and cytokeratins[16,17].

The CellSearch CTCs system (Veridex) is commonly used, and in today's scenario it is the gold standard and the only FDA-approved method for CTC detection. It was approved in 2004 for extracting CTCs in metastatic breast cancer[18] and later in colorectal[19] and prostate cancers[20]. Equipment cost ranges from 600000-800000 USD.

The CellSearch CTCs Kit is generally used for the enumerization of CTCs of epithelial origin (CD45-, EpCAM+, and cytokeratins 8+, 18+, and/or 19+) from whole blood sample and works on the principle of anti-EpCAM immunomagnetic

Table 1 Techniques for circulating tumour cell isolation, markers, and their limitations

No.	Name	Property	Markers	Limitations	Ref.
1	CellSearch	Isolation by anti-EpCAM antibody coated immunomagnetic beads	EpCAM, CKs, CD45, DAPI	Only suitable for cancer of epithelial origin but not for that undergoing the EMT Cells are not viable after detection	[48]
2	AdnaTest	Separation by way of anti-EpCAM and anti-MUC1 antibody coated immunomagnetic beads	EpCAM, MUC1, mucin-1, HER2	Possible false-positive finding due to expression of a selection marker being present in other cells other than CTCs Cells are not viable after detection	[49]
3	MACS	Immunomagnetic CTC enrichment by antibodies against cell surface markers	CK19, EpCAM, Her-2, MUC-1 CK7, CK8, CK18, CK19	Lengthy processing time and low sensitivity	[50]
4	MagSweeper (Illumina Inc)	Immunomagnetic isolation of CTC by antibodies against EpCAM and cellsurface markers Captured cells are viable with intact RNA	EpCAM, CD45, DAPI	Less sensitive during the early stages of tumour development	[51]
5	CTC Chip	Utilizes bifurcating traps to capture CTCs, release *via* flow reversal	EpCAM, CKs, CD45, DAPI	Identification of CTCs is lower than other methods	[52]
6	GEM chip	Geometrically enhanced mixing chip structure that allows enhanced capture of CTC on antibody coated surfaces	EpCAM, DAPI, CD45, cytokeratin	Low sensitivity	[53]
7	Onco Quick (Greiner BioOne, Frickenhausen, Germany)	Separation of erythrocytes and some leukocytes from CTC. High sensitivity, Quantification	CCNE2, DKFZp762E1312, EMP2	No morphology confirmation; not really capture CTCs	[54]
8	ISET (Rarecells Diagnostics)	Rapid processing; non-antigen dependent; Filter based approach	CKs, EGFR, VE-cadherin, Ki67	Size-dependent, manual processing	[55]
9	EPISPOT	Removes leukocytes *via* CD45 depletion Can detect viable CTCs	CD45, CK19, mucin-1, cathepsin-D	Problem arises when antigen levels are lower or binding efficiency is reduced	[56]
10	Ficoll + RT-PCR	Separation of CTC based on size dependent enrichment. High Sensitivity	CK-19, HER2, h-MAM, CEA, maspin, GABA A, B726P	No morphology confirmation	[57]
11	Cyttel Method	Negative immune-magnetic selection of WBC (CD45 antibody)-High detection rate	CD45	-	[58]
12	MetaCell	Size-based enrichment and separation for viable CTCs	CK-18, -19, -20, CK-7, EPCAM, MUC1, HER2, EGFR	Lengthy processing time	[59]

MACS: Magnetic-activated Cell Sorting; CTC: Circulating tumour cell; RT-PCR: Real-time polymerase chain reaction.

enrichment. For the CTC enumeration step, the CTC kit has reagents to stain and fix the cells. The protocol has been described in detail by Coumans and Terstappen[21]; EpCAM is unique for epithelial cells and is expressed in most carcinomas in a very strong manner, while its expression is limited to embryonic stem cells in non-epithelial cells[22]. However, EpCAM is not a universal cancer marker. EpCAM expression is quite absent in squamous carcinoma or down-regulated if cancer cells undergo epithelial-to-mesenchymal transition (EMT); such cancer cells can escape the capturing process. A gene expression study on breast cancer showed that EpCAM was down-regulated in mesenchymal lines relative to the epithelial cell lines[23] and EMT-induced breast cancer cells[24].

Despite its high specificity and efficiency, some of the disadvantages of the CellSearch system are: (1) It is only suitable for cancer of epithelial origin but not for cancer cells undergoing EMT; (2) CTCs cannot be further analysed in real-time and live-cell conditions, because CTCs cannot be kept alive for a long time; and (3) The use of expensive antibodies leads to high detection cost.

Other label dependent methods

AdnaTest is the second most common method used for CTC detection after the CellSearch. It is a commercially available positive selection method in which immuno-

magnetic beads are coated with a combination of antibodies for the increased capture and enrichment of CTCs. Through gene expression testing of specific tumour markers in the captured cells and comparison of this with their primary and metastatic tumour equivalents, clinicians may analyze the clinical implications of CTCs. Therefore, it has both diagnostic and prognostic value. CTCs captured by magnetic beads coated with antibodies (EpCAM, MUC-1, *etc*.) are then analyzed by multiplex real-time polymerase chain reaction (RT-PCR) gene panels.

The other techniques which are used for CTC enrichment are as follow: (1) Magnetic-activated cell sorting system: This system works on immunomagnetic CTC enrichment by antibodies against cell surface markers. Magnetic-activated cell sorting offers both positive and negative enrichment for the high-efficient and accurate isolation of CTCs (Clinical value: Prognosis and diagnosis); (2) MagSweeper: This system works on immunomagnetic isolation of CTCs by antibodies against EpCAM and other cell surface markers. It can process large amount of blood (approximately 9 mL/h) and can detect 1–3 CTCs *per* 1 mL of whole blood (Clinical value: Prognosis); (3) GEM chip: This is geometrically enhanced mixing chip that permits increased identification of CTCs on antibody-coated surfaces (Clinical value: Treatment monitoring and prognosis); (4) Onco cell enrichment and extraction: This platform uses microfluidic chip with internal surfaces functionalized with an antibodies group against bio-tumour-associated and mesenchymal markers (Clinical value: Treatment monitoring, prognosis, and diagnosis); (5) Graphene oxide chip: In this platform, graphene oxides (GO) nanosheets are used to capture antibodies against cell surface markers of CTCs with a high sensitivity (Clinical value: Prognosis); (6) Ephesia (CTC-chip): Micromagnetic particles are functionalized with EpCAM antibodies which can be self-assembled in a micro-fluidic platform (Clinical value: Prognosis and diagnosis); (7) Quadrupole magnetic separator: This separator works as negative CTC enrichment after it combines with viscous flow stress and magnetic force for the recovery of unlabelled CTCs (Clinical value: Treatment monitoring, prognosis, and diagnosis); and (8) CTC-iChip: This chip works on lateral displacement, inertial focusing, and magnetophoresis for fast isolation of leukocytes by using anti-CD45 and anti-CD66B antibodies in negative enrichment or EpCAM activated beads for CTC enrichment in positive enrichment of CTCs (Clinical value: Prognosis and diagnosis).

Label independent methods

Many newly studied methods forCTC recognition have been reported[25]. Separation of circulating tumor cells by physical properties, *i.e.*, density gradients and gravity, using microfluidic technology[26,27] have been found to be able to capture CTCs efficiently.

The different tools and techniques described for CTC isolation in this category are as follows: (1) ISET: Filter based isolation and enrichment (Clinical value: Treatment regimen and prognosis); (2) MetaCell system: Size-based enrichment and separation (Clinical value: Diagnosis and prognosis); (3) Parylene filter: Filter based isolation and enrichment (Clinical value: Diagnosis and prognosis); (4) ScreenCellCyto: Filter based size-exclusion separation and enrichment (Clinical value: Diagnosis); (5) Cell sieve: Micofilter based isolation and enrichment (Clinical value: Diagnosis and prognosis); (6) Parsorti technology: Micro fluidic separation of CTC based on their size and deformability (Clinical value: Diagnosis and prognosis); (7) RosetteSep CTC enrichment/CD45 depletion: This is an immuno-density negative selection method for CTCs using tetrameric antibody complexes that identify CD45, CD66 ,and glycophorin on WBCs and red blood cells (RBCs) (Clinical value: Prognosis); (8) Onco Quick: Isolation of RBCs and some leukocytes from CTCs by using filtration through porous membrane followed by density-gradient centrifugation for better CTC enrichment (Clinical value: Prognosis); (9) Cyttel method: Based on the negative immuno-magnetic selection of WBCs (antibody CD45) followed by gradient centrifugation and smearing through slides of isolated CTCs (Clinical value: Prognosis and treatment regimen); (10) AccuCyte-CyteFinder: Automated rapid imaging of single rare cells in CTCs, followed by density-based cell separation method (Clinical value: Prognosis); (11) EPISPOT: Negative enrichment using CD45 depletion (Clinical value: Prognosis); (12) Cyto Track: Use of fluorescently labeled cells against EpCAM and scanned with the help of beam (Clinical value: Prognosis); (13) Fiber optic array scanning technology (FAST) (Clinical value: Prognosis); (14) Image Stream: Immunogenetic sorting of blood followed by flow cytometry and enumeration of CTCs by fluorescent microscopy (Clinical value: Diagnosis); (15) DEPArray: Moving dielectrophoretic cages for cell capture coupled with Sanger sequencing (Clinical value: Tumour monitoring and prognosis); (16) Vortex: CTC extraction using microscale vortices and inertial focusing (Clinical value: Diagnosis, prognosis, and treatment planning); (17) ClearCell FX: CTC

separation based on size using Dean Flow Fractionation (Clinical value: Diagnosis); and (18) qRT-PCR: Separation of CTCs based on size-dependent enrichment using CD45, CK19, and CK20 (Clinical value: Prognosis).

COMPARISON OF CELLSEARCH SYSTEM WITH OTHER TECHNIQUES

The high sensitivity and specificity of CTC detection methods have a great effect in improving patient outcomes. Politaki *et al*[28] have compared CTC detection rates and prognostic significance in breast cancer patients by comparing three commonly used methods including CellSearch, qRT-PCR, and double immunofluorescence (IF) microscopy. They analyzed early diagnosed (n = 200) and metastatic (n = 164) breast cancer patients before the start of adjuvant or first-line chemotherapy. They compared CellSearch system, qRT-PCR for *CK19* mRNA detection, and double IF microscopy by using A45-B/B3 and CD45 antibodies and concluded that patients were more likely to be CTC-positive using the CellSearch (37%) than qRT–PCR (37% *vs* 18.0%, P < 0.001) or IF (37% *vs* 16.9%, P < 0.001). In another study[29], CellSearch was compared with Adna Test and RT-PCR in breast cancer, and it was found that multimarker qRT-PCR showed a superior sensitivity for the detection of CTCs in metastatic breast cancer patients compared with the CellSearch system and the AdnaTest. There is limitation of the assessment by PCR as it provides the number of target transcripts based on the actual number of CTCs present in a sample[30] and does not allow the morphological assessment of cells. Two cell-based detection assays, the CellSearch and Onco-Quick (for density gradient centrifugation), on comparison revealed that the CellSearch was a far more accurate and sensitive method to detect and enumerate CTCs[31].

There is one study by Gervasoni *et al*[32], in which they compared the capacity of three methods, multimarker RT-PCR assay, standardized CellSearch method, and dHPLC-based gene mutation analysis, to detect CTCs in the blood of 20 CRC patients (stage I = 5, stage II = 8, stage III = 6, and stage IV = 1). They found CTC positivity in 75% of samples by RT-PCR, 20% by CellSearch method, and only 14.3% of samples were found to be gene mutated with the presence of CTCs by HPLC method. These results show that out of these three methods tested, multimarker RT-PCR assay provides the maximum probability of CTC detection. Future studies, by using the above three distinct methods for follow-up, may provide more information about the prognostic significance of CTCs detected through single method assay *vs* combination of different assays[32].

CIRCULATING TUMOUR CELLS AND THEIR CLINICAL APPLICATIONS IN COLORECTAL CANCER

CTC characterization and number may be useful in several ways where they can be used both as a prognostic marker for survival as well as prediction of response to cancer treatment[33]. A multivariate analysis[34] demonstrated that CTC count is the strongest prognostic biomarker for patient survival. If the CTC number increases or remains static, the treatment can be deemed to be ineffective, whereas, if CTC number decreases, the treatment may be effective. Several studies have shown that the presence of as few as 3 to 5 CTCs in 7.5 mL of blood is associated with poor PFS and OS rates[35]. Studies with the CellSearch system and others have shown that high numbers of CTCs are associated with lower DS and OS rates[36]. In a study of 413 metastatic CRC patients being treated with first, second, or third-line therapy, patients with a baseline CTC number of more than 3/7.5 mL had significantly poor median PFS (4.4 mo *vs* 7.8 mo, P = 0.004) and OS (9.4 mo *vs* 20.6 mo, P < 0.0001) compared with patients with less than 3 CTCs/7.5 mL[37,38]. CTC evaluation, during treatment, may be used as a prognostic predictive marker to determine progression-free survival (PFS) and OS. The CellSearch system has its own limitation; the method of isolation utilizes EpCAM expression on the cell surface of the tumour, which is expressed in 75% of cancer types. A study by Fang *et al*[39] (2016) analyzed the expression of cell surface markers CD133, CD54, and CD44 with the help of flow cytometry to analyze the correlation between cellular subpopulations and colorectal liver metastasis. They observed that the expression of cellular subpopulations (CD133+, CD54+, and CD44+) was higher in the peripheral blood of CRC liver metastasis in comparison with those with no metastasis (P < 0.001). In a study by Lalmahomed *et al*[40] (2015) on peripheral blood of 151 CRC patients who underwent liver metastasectomy, CTCs were detected

by the CellSearch system after a density-gradient-based enrichment step. They found that CTCs were detected in 75 samples (43%), out of which 16% had 3 CTCs/7.5 mL of blood. Patients with or without detectable CTCs have an almost similar 1-year recurrence rate (47% *vs* 48%, respectively). A similar recurrence rate was also reported with low *vs* high CTC count (< 3 or 3 CTCs/7.5 mL of blood: 50% *vs* 47%, respectively). In their report, no difference was found in disease-free survival and OS among patients with or without CTCs. A report by Shimada *et al*[41] (2012) found that detecting CEA/CK/CD133 mRNA in tumour drainage blood (RT-PCR method) could act as a prognostic marker in patients with Duke's stages B and C CRC. The findings of the CTC isolation techniques and their clinical significance have been given in detail in Table 2. Hendricks *et al*[42] (2020) used qRT-PCR for indirect CTC detection, which was already applied in previous studies on CRC patients and found to have prognostic value. An earlier study by Sastre *et al*[43] (2008) reported that the CellSearch system could identify CTCs in CRC patients and that CTC positive cases were correlated with the stage of the disease (P = 0.005) but there was no significant correlation between CEA levels, tumour locations, grade of differentiation, and lactate dehydrogenase (LDH) levels. A meta-analysis by Katsuno *et al*[44] (2008) of a total of nine studies found that CTC-positive patients (in blood samples by RT-PCR), correlated with lymph node (LN)-positive patients (50%) *vs* LN-negative patients (21%).

Guadagni *et al*[45] (2020) have published a couple of studies about the role of CTC based therapeutic decision making in CRC. In the first study[45], they included 62 patients with advanced unresectable rectal cancer and reported that where the patients were selected for the treatment based on CTCs (HPP/target-therapy group, n = 43); the disease control rate was significantly higher (PFS = 8 mo, OS = 20 mo) as compared to those given systemic chemotherapy (n = 19) based on age, co-morbidity, and performance status (PFS = 4 mo, OS = 8 mo). The second study[46] was performed on 106 advanced unresectable CRC patients. The therapy was decided based on CTCs (HAI/targeted, n = 44), age, and co-morbidity performance status (systemic chemotherapy, n = 62). The authors found that the group where treatment was given based on CTCs had longer PFS and median survival (MS) (PFS = 5 mo, MS = 14 mo) as compared to those given therapy based on age and co-morbidity performance status (PFS = 3 mo, MS = 8.5). Finally, they concluded that CTCs can be used to choose therapeutic options in unresectable CRC.

Inherited or acquired resistance in response to specific treatment can be assessed with CTCs which may also work as pharmacodynamic markers. CTCs have enhanced our knowledge and understanding about the primary mechanisms of cancer metastasis. This understanding may be useful in therapeutic manipulation with the help of new targets. CTCs were evaluated in phase I trial based on their count and the expression of insulin-like growth factor-1 receptor (IGF-1R) to find out their therapeutic applications. The CellSearch system was used, either alone or in combination with docetaxel, to count CTCs in patients treated with monoclonal antibodies against IGF-1R. Positive IGF-1R and CTC response was seen in 23 out of 26 patients. These patients responded better in case of combined treatment than in case of the remaining three patients who were negative for IGF-1R. From these findings, it was concluded that CTCs can be used as a potential marker for the selection of chemotherapy[47].

CHALLENGES IN CIRCULATING TUMOUR CELL IDENTIFICATION

CTC interpretation is quite promising but has limitations such as factors like requirement of large volume of blood, small size of the cancer patient population, and the standard value for comparison (*i.e.*, CellSearch, blood sample, other micro-devices, *etc.*). Till now, many reports have enlightened the prospects for cancer patient monitoring, and for few years researchers have focused on CTCs to explore their biological metastatic property and role in cancer treatment monitoring. Among the several important clinical applications for CTC technology is the correlation of CTC count with OS and PFS as a measure of clinical outcome.

The presence of CTCs in the blood sample is also a major challenge. If they are present, their heterogeneity of unknown extent is also present. Because of this nature, it demands an ongoing diversity in the detection and characterization of CTCs using the present available and upcoming methods in the future.

Table 2 Studies showing postoperative isolation of circulating tumour cells in colorectal cancer–markers, techniques, and clinical implications

No.	Technology	Markers	Number of patients	TNM stage	Correlation	Clinical significance	Ref.
1	CellSearch system	EpCAM	164	I-III	With stage	N/A	[60]
		EpCAM	24	IV	With therapy response	May be used in monitoring response to therapy	[61]
		EpCAM	97	II	With stage	Correlates with stage	[62]
		CD133+, CD54+, CD44+	15 (nmCRC); 95 (mCRC)	I-IV	≥ 5 CTCs were 8 times more likely to develop distant metastasis. CTC counts show good correlation with colorectal neoplasm	Independent prognostic marker for nmCRC	[63]
		hTERT, CK19, CK20, CEA	438	I-III	-	Poor relapse free survival	[64]
		hTERT, CK19, CK20, CEA	157	I-III	With stage	Poor relapse free survival and overall survival	[65]
		Survivin, CK20 and CEA	156	I-III	With stages (Duke's) and lymph node metastasis.	Useful as an adjunct in detection of CRC patients	[66]
		CD133, CEA, CK20, CK19,	197	II-III	CEA/CK/CD133 expression and stage (Duke's)	Prognostic significance (Duke's stages B and C)	[44]
		hTERT, CK-19, CK-20, CEA, GAPDH and mRNA	72	I-IV	CEA, mRNA: With stage, vascular invasion, and postoperative metastasis	Prognostic and predictive	[67]
2	Flow-cytometry with immunofluorescence	CTCs	18	I-III	With stage and also detected in an early cancer stage.	Predictive	[68]
3	Pyrosequencing	KRAS (Codon 12/13)	26	IV	No association	Prognostic	[69]
4	MetaCell separation method	CTCs	98	I-IV	CTC-positive in 83% CTC-negative in 17%	Prognosis and predictive	[70]
5	Mag Sweeper	PIK3CA	242	-	Mutational discordance found between CTCs, DTCs, and metastases, and among CTCs; DTCs from this patient propagated *in vitro* contained a PIK3CA mutation	Investigating new drug therapies	[71]
6	CTC-Chip	EpCAM, HER2, and EGFR	-	-	Efficiency of 87.5%	*In situ* protein expression, and culture CTCs from the same set of cells	[72]

CRC: Colorectal cancer; DTC: Disseminated tumour cells; GAPDH: Glyceraldehydes 3-phosphate dehydrogenase; nmCRC: Non-metastatic CRC; mCRC: Metastatic CRC.

CONCLUSION

CTCs have become a hot pursuit and in recent years many new CTC detection technologies have emerged. Discoveries of these technologies from laboratory to clinical practice are non-trivial. Only a few systems are available for routine use in the clinical setting, but not freely available. CTC detection is challenging because of the small number of circulating cells but has been found both in metastatic and non-metastatic cancer (Table 3). It has been well correlated with the stage of the disease, prognosis, and survival but has a limited role in therapeutic decision-making. There is a need for the development of newer, cheaper techniques of CTC detection which can be used as an alternative to invasive diagnosis and treatment monitoring. Future research is required as the current literature has limited information on its use in routine clinical practice but the future is promising.

Table 3 Circulating tumour cells in metastatic *vs* non-metastatic colorectal cancer

No.	Type of CRC	Markers used	Detection method used	Relevance	Clinical implications	Limitations of the study	Ref.
1	nmCRC	CEA, CA19-9, CA72-4	Cyttel	Diagnostic/prognostic/predictive	Combination of CTCs and CEA: Diagnostic and prognostic indicators	Small sample size, weak power of the study	[73]
2	mCRC	CK, CD45	Immunomagnetic separation	Prognostic/predictive	The number of CTCs before and during treatment is an independent predictor of PFS and OS in patients with mCRC	The baseline unfavourable CTC was low (26%) and overall CTC yield was less than in other epithelial cells	[74]
3	mCRC	ALDH1, CD44, CD133, MRP5, Survivin	qRT-PCR	Prognostic	Poor prognosis and chemo therapy non-responsiveness Survivin and MRP5 selection of mCRC patients resistant to 5-FU and L-OHP	Require further molecular analyses of CTCs for selection of targeted agents	[75]
4	mCRC	CEA	Cyttel method, immunofluorescence *in situ* hybridization technologies (imFISH)	Prognostic	PFS, OS	Small sample size Lack of dynamic enumeration of CTCs	[6]
5	mCRC	VEGF, CD133+, CD34+/KDR + EPC, CD-34 ¯VEGFR2	Flow cytometry/IHC	Prognostic	Treatment response; PFS, OS	-	[76]
6	nmCRC	CD133, CD166, CD44, EpCAM, ALDH1	Tissue microarray, IHC	Prognostic	No association with poor clinical response; OS	Treatment information was missing (local recurrence, distant metastasis, and postoperative therapy)	[77]
7	nmCRC	CK19, MUC1, CD44, CD133, ALDH1	Flow-cytometry, CellSearch, Cytomorphology, qPCR	Prognostic	May be useful as a therapeutic target; PFS, OS	-	[78]

CRC: Colorectal cancer; nmCRC: Non-metastatic CRC; mCRC: Metastatic CRC; OS: Overall survival; PFS: Progression-free survival; IHC: Immunohistochemistry.

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MINIREVIEWS

Immunotherapy combinations and chemotherapy sparing schemes in first line non-small cell lung cancer

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Abstract

In recent years, studies have explored different combinations of immunotherapy and chemotherapy. The rationale behind these is the improved survival outcomes of new immunologic therapies used in first-line-treatment of advanced non-small cell lung cancer. Moreover, for the most-studied combinations of anti-programed death-1 (PD-1)/programed death ligand-1 (PD-L1) with the addition of platinum-based chemotherapy, recent research is investigating whether combining different immunologic antitumoral mechanisms of action, such as anti-PD-1/PD-L1 and anti-CTLA-4, or anti-PD-L1 and anti-TIGIT, with or without chemotherapy, can improve efficacy outcomes compared with more classical combinations, or compared with standard chemotherapy alone. Here, we present the data of the

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main randomized studies that have evaluated these combinations, focusing on the basic rationale behind the different combinations, and the efficacy and tolerability data available to date.

Key Words: Immunotherapy combinations; Anti- programmed cell death protein; Anti-programmed cell death ligand 1; anti-TIGIT; anti-CTLA-4; Combo; Non-small cell lung cancer

Core Tip: This review presents the results of the main articles reporting on immunotherapy combinations, with or without chemotherapy, focusing principally on efficacy and toxicity data. The convenience of adding shorter chemotherapy regimens *vs* the standard 4-cycle regimens to immunotherapy doublets is discussed.

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INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths worldwide. An increased knowledge of lung cancer pathology as well as of tumoral immunity has permitted the discovery of different targeted molecular treatments and immune-therapies involving an important change of the lung cancer paradigm[1].

Anti-programed death-1/programed death ligand-1 (PD-1/PD-L1) treatment, recently approved in advanced lung cancer treatment, is an important immune checkpoint of anti-tumor activity in our immune system. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is another component of these control immune checkpoints and different inhibitors have been developed to look their role in tumor recognition[2] (Figures 1 and 2).

Non-small cell lung cancer (NSCLC) has a high level of heterogeneity secondary to a different molecular immune subtype and a complex immune environment. This complexity justifies heterogenous responses to immunotherapy (IT) and their combinations[2].

The administration of mono-IT (m-IT) with anti-PD-L1/PD-1, or a combination of this type of agent with platinum-based chemotherapy (CT), is the standard therapy for the different subtypes of non-small cell lung cancer without treatable mutations. Several studies focusing on combinations of different checkpoint inhibitors, such as anti-PD-1/PD-L1 and anti-CTLA-4, have shown these to be advantageous compared with CT alone[3,4]. The association of these IT combinations with CT has also been analyzed in two phase III studies [Checkmate 9LA study (CM9LA) and CCTG BR.34], with interesting results in favor of the new combinations[5,6]. The CYTISCAPE study, presented recently in ASCO 2020, proposes a combination of anti-PD-L1 and anti- T-cell immunoreceptor with Ig and ITIM domains (TIGIT), a new checkpoint inhibitor present in activated T-cells. This phase II study reported favorable outcomes for this IT doublet (d-IT) in the population with PD-L1 > 1% *vs* monotherapy with anti-PD-L1[7].

Here, we briefly summarize the most significant aspects of these new combinations.

BIOLOGICAL BASIS OF IT COMBINATIONS

The biological rationale for combining IT is based on the complexity of immune surveillance regulation, the effect of other anti-tumor treatments on tumor immunogenicity, and the non- redundancy of immune checkpoint regulation[8].

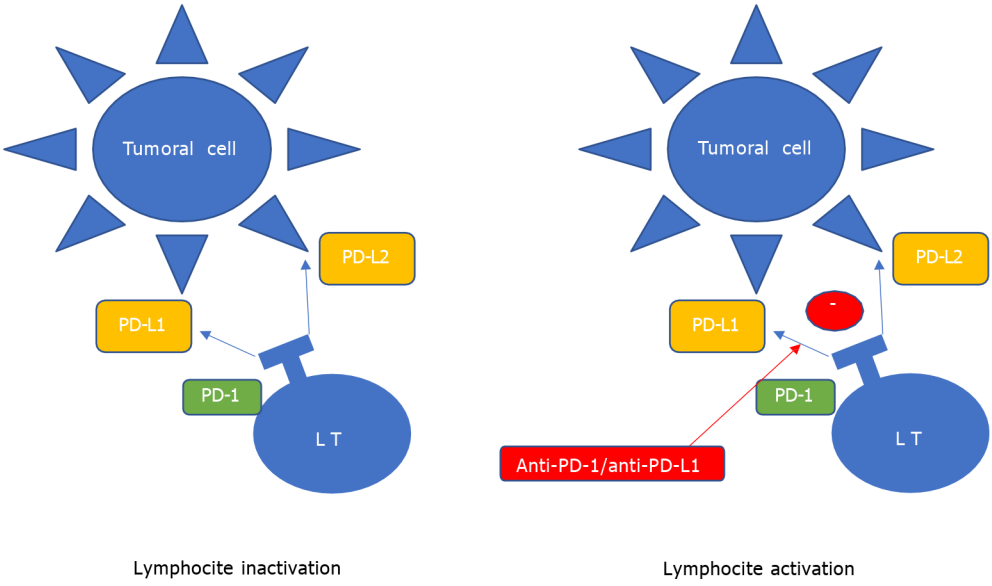

Figure 1 A schematic diagram showing programed death-1/ programed death ligand-1 interaction with immune cells in cancer. PD-1: Programed death-1; PD-LI: Programed death ligand-1.

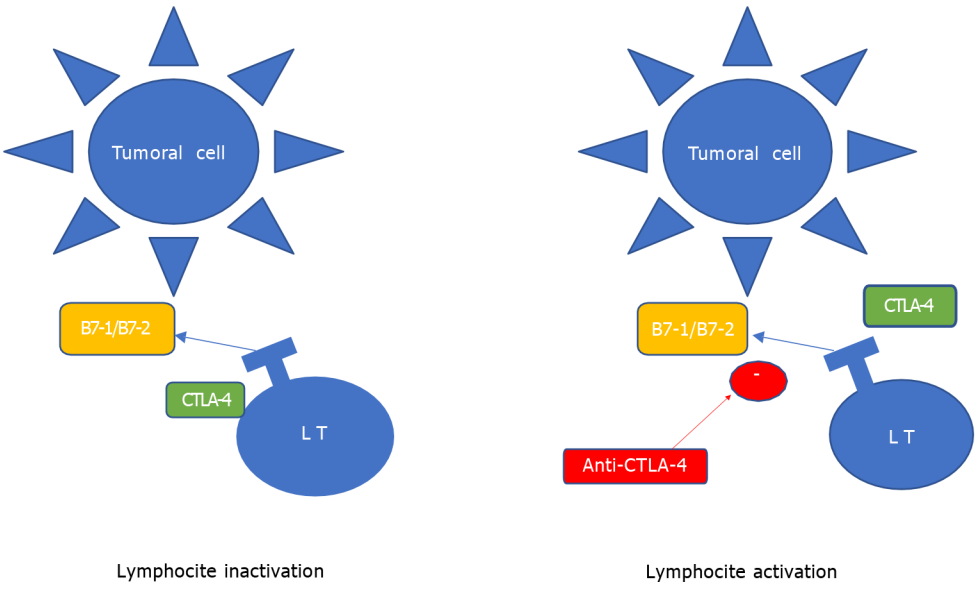

Figure 2 A schematic diagram showing cytotoxic T lymphocyte–associated antigen 4-2/B7-1-B7-2 interaction with immune cells in cancer. CTLA-4: Cytotoxic T lymphocyte–associated antigen 4.

Most chemotherapeutic agents are regarded as immunosuppressants, exerted this effect by a direct inhibition or killing of effector cells, as well as indirectly by inducing anergy or immune paralysis[9]. However, combinations of CT and ICI have a proven synergistic effect: CT aids in fast tumor regression, and a reduction in tumor burden and immune checkpoint blockade may prolong this effect, thus inducing a long-lasting anti-tumor response[10]. In addition, the non-overlapping toxicity profiles of IT and CT render them good candidates for combination strategies.

In addition to immune regulation, angiogenesis is another hallmark of cancer evolution, promoting tumor vascularity to sustain cancer cell metabolism and invasive capacity, and also favors cancer immune escape. Vascular endothelial growth factor, one of the main regulators of angiogenesis, can also induce T-reg cells and inhibit dendritic cell and T-cell maturation, thus inducing an immunosuppressive status, lymphocyte migration to the tumor site, and negatively regulating immune priming [11,12]. Combining anti-angiogenics with immune checkpoint inhibitors could thus be synergic in reducing tumor vascularization and decreasing the immune suppressive microenvironment. It may also have a synergistic effect by boosting tumor-specific

immunity.

Immune checkpoints exert a negative regulatory effect on CD8+ T cells by interacting with their ligands expressed on different cells, such as APC or T cells. Preclinical data indicate that the combination of PD-1 and CTLA-4 receptor blockade may improve antitumor activity. In vitro combinations of nivolumab plus ipilimumab increase IFN-γ production 2- to 7-fold over either agent alone in a mixed lymphocyte reaction (Table 1). The combination was also observed to exert an increased antitumor activity in 3 of 5 syngeneic murine cancer models. In a murine melanoma vaccine model, blockade with either CTLA-4 or PD-1 antibodies increased the proportion of CTLA-4 and PD-1-expressing CD4/CD8 tumor infiltrating T effector cells, and dual blockade increased tumor infiltration of T effector cells and decreased intratumoral T regulatory cells, compared with either agent alone[13].

TIGIT is a novel immune inhibitory receptor. It is a member of the immunoglobulin superfamily and is expressed in a wide variety of human tumors. TIGIT is expressed on the surface of activated T-cell and natural killer (NK)-cell subsets and interacts with high affinity with CD155 [also known as poliovirus receptor (PVR)]. It is also highly correlated with T-cell infiltration and PD-1 expression[14]. Genetic ablation of TIGIT in T cells in mice results in exacerbated T-cell responses in preclinical models of autoimmune diseases and viral infections, demonstrating the role of TIGIT in the inhibition of T-cell responses. Because TIGIT and PD-1 are coordinately expressed by tumor-infiltrating T cells in several human tumors, inhibition of the TIGIT/PVR pathway may complement and potentiate the anti-tumor activity of a PD-L1 pathway inhibitor. In preclinical models, concomitant blockade of both TIGIT and PD-L1/PD-1 pathways demonstrated superior efficacy over the respective single-agent treatments [15].

In conclusion, based on preclinical and clinical studies, there is a strong rationale for combining IT and other agents, and this potentially synergistic effect has been demonstrated with improved outcomes in the clinical setting.

PHASE I-III STUDIES

As explained previously, there is a clear justification for combining different therapeutic strategies. Consequently, several studies have been designed to explore the clinical efficacy of these combinations in the context of advanced NSCLC.

Since pembrolizumab was first approved in first-line therapy of patients with PDL1 > 50%, several studies, which we describe below, have analyzed the combination of anti PD-1/PDL-1 and anti-CTLA-4, with or without the addition of CT[14] (Table 2).

Checkmate 012 (CM012) was the first phase I study to explore the combination of ipilimumab-nivolumab (NI) *vs* nivolumab (N) in CT-naïve patients with advanced NSCLC. This study assessed three alternative regimens with different doses of I and N, with 6 weekly *vs* 12 weekly schemes of I, with tolerability as the primary endpoint. This study confirmed the safety of the combination, predicting a high response rate and improved survival (OS)[17].

With the data from CM012, Checkmate 227 (CM227), a phase III study was proposed. This study was comprised of three parts: Part1A that assessed the efficacy of the combination of NI *vs* N in monotherapy *vs* standard CT in patients with negative PD-L1 expression, part 1B that compared the combination of NI *vs* N + CT *vs* standard CT in PD-L 1 negative patients, and part 2 which evaluated the efficacy of the combination of N + CT, regardless of histology and PD-L1 expression. The study reported a global benefit for the NI doublet in OS, independently of PD-L1 [17.1 ms *vs* 13.9 ms, hazard ratio (HR) 0.73; 95%CI: 0.64-0.84]. When these data are analyzed in relation to PD-L1, several important observations can be made: in the PD-L1 negative group the benefit in OS in favor of the IT doublet was maintained at 17.2 *vs* 12.2 mo (HR 0.62, 95%CI: 0.48-0.78). In the PD-L1 positive group (≥ 1%), OS was also better with the combined IT treatment (17.1 ms *vs* 14.9 ms, HR 0.79; 95%CI: 0.65-0.96). Noteworthily, in this group with 1-49% PD-L1 expression, no differences in OS were found. Also in this study, data of levels of tumor mutational burden (TMB) were reported early on. Hence, in the high TMB group (> 10 mut/Mb), a statistically significant benefit in favor of the combination was reported. Finally, the results of part 2 were communicated, in which the primary final endpoint of OS for N-CT was not reached in patients with non-squamous NSCLC[3].

The CM9LA is a phase III trial with a novel design that adds two cycles of CT to the NI doublet. In the control arm, the CT regimen preferred by the clinician was established, or maintenance with permetrexed in non-squamous histology. With a

Table 1 Main programed death ligand-1 and cytotoxic T lymphocyte–associated antigen 4 pathways

Checkpoint pathways	Anti-PD-L1 pathway	Anti-CTLA-4 pathway
Receptor expression	Activated T-cells	Activated T-cells, B-cells and NK cells
Ligands	B 7.1 (CD80); B7.2 (CD86)	PD-L1 (B7-H1), PD-L2 (B7-DC)
Mechanism of immune modulation	T-cell activation at initial stage; Competition with co-stimulatory receptor CD-28 for ligand binding; Down regulation of helper T cells CD4 activity; Enhancement Tregs-cells immunosuppressive activity	Suppresses activated T cells in tissues and tumor environment; Express in Tregs-cells may enhance immunosuppressive activity; Limits of B-cells and NK- cells activity; PD-L1 interact with CD-80 to down-modulate T-cells activity

NK: Natural killer; PD-L1: Programed death ligand-1; CTLA-4: Cytotoxic T lymphocyte–associated antigen 4.

Table 2 Summary of the main Phase III studies of IT combinations

Trial	Ph[1]	Drugs	Endpoints/outcome	PD-L1/TMB
				Population
				Subgroups
Checkmate 227	III	NI *vs* N *vs* CT[1]	OS PD-L1 > 1% and in non-selected population	PD-L1 ≥ 1%: NI 17 m *vs* CT 14.9 m, *P* = 0.007
			NI *vs* CT	PD-L1 < 1%: NI 17 m *vs* CT 12.2 m, *P* =0.007
			17 m *vs* 13.9 m	
MYSTIC	III	DT *vs* D *vs* CT[1]	OS D *vs* CT OS and PFS DT *vs* CT PD-L1 > 25% population	PD-L1 > 25%: D *vs* CT[1]
				16 m *vs* 12 m, *P* = 0.04
				DT *vs* CT[1] 11.9 *vs* 12 m, *P* = 0.2
				TMB > 20
				DT *vs* CT[1] 21 m *vs* 10 m
Checkmate9LA	III	NI + CT[1] X2 *vs* CT[1] X4	OS in non selected population	NA
			OS NI-CT 15.6 m *vs* CT 10.9 m, *P* = 0.0006	
CCTG BR.34	III	DT+ CT[1] x 4 *vs* Durvalumab + tremelimumab (DT)	OS in non selected population	PD-L1 TPS ≥ 50%, DT-CT *vs* DT (HR 0.64, 95%CI: 0.40-1.04, *P* = 0.07). Plasma TMB < 20 mut/Mb was associated with shorter survival in both treatment groups (HR 1.99, 95%CI: 1.3-3.1)
			16.6 m DT-CT *vs* 14 m DT	
CITYSCAPE	II	TA *vs* PA	ORR and PFS en PD-L1 > 1% PFS TA 7.7 m *vs* PA 3.2 m	PD-L1 > 50%
			ORR TA 37% *vs* PA 20%	PFS TA NA *vs* PA 4.1
				ORR TA 66 % *vs* PA 24%

[1]Ph; Phase.
NI: Nivolumab + Ipilimumab; N: Nivo; DT: Tremelimumab + Durvalumab; D: Durvalumab; PD-1: Programed death-1; TMB: Tumor mutational burden; HR: Hazard ratio; CI: Confidence interval; TA: Tiragolumab + atezolizumab; PA: Placebo + atezolizumab; NA: Not available; CT: Chemotherapy.

follow up of 12.7 mo, the experimental arm showed a significant improvement in OS in all subgroups (15.6 ms *vs* 10.9 ms, HR 0.66; 95%CI: 0.55-0.8)[5].

The combination of Durvalumab (D) and Tremelimumab (T) has also been explored in different phase I-III studies. One of the first phase III trials to analyze this combination in NSCLC was the ARTIC study. This was comprised of two independent sub-studies: one focused on the PD-L1 > 25% population, comparing D *vs* standard CT in patients pre-treated with platinum, and another in the population with PD-L1 < 25% with three arms: DT *vs* D *vs* T in the same patient profile. In both sub-studies, the data from the D and DT arms were more favorable than CT alone[18]. Continuing with this combination of DT in first-line, the MYSTIC phase III study[4] once again focused

on the population of PDL-1 > 25%. The patients were randomized to receive D *vs* DT *vs* platinum-based CT, in this case in first-line of NSCLC. The efficacy data for the mono-IT *vs* CT showed a non-significant superiority for the former (16 mo *vs* 12 mo), although in the d-IT the median OS of 11.9 mo was shorter than that obtained with CT. No benefit in favor of IT was found for PFS either. However, in this study TMB was included as a predictive biomarker of response to IT, with the subgroup TMB > 20 presenting an OS in favor of the IT doublet (21.9 mo *vs* 10 mo).

Continuing to focus on the DT combination, CGC BR.34, another phase III study emerged, in parallel with CM9LA, to address the need to explore the efficacy of d-IT. In this work, 4 CT cycles (platinum-pemetrexed) were added to the DT doublet in first-line therapy in a non-squamous population. Maintenance treatment was allowed in both arms. After a six-month follow up, no differences were found in OS, although the analysis of PFS revealed statistically significant differences in favor of the combined treatment of IT-CT (7.7 mo *vs* 3.2 mo)[6].

CITYSCAPE is a phase II trial that evaluates the possible efficacy of combining Tiragolumab (Ti) (an inhibitor of the immune modulatory receptor TIGIT) with Atezolizumab (A) in first-line of NSCLC with PDL > 1%. Preliminary phase I studies (GO 30103) showed the benefit of combining TIGIT inhibition and anti-PD-L1, revealing a greater response rate in pre-treated patients. The first data from this study were presented in ASCO 2020, and the preliminary analysis was positive in favor of the combined approach (TiA *vs* A-placebo) (5.6 ms *vs* 3.9 ms, HR 0.58; 95%CI: 0.38-0.89). Moreover, an exploratory analysis in patients with high PD-L1 expression, or with a tumor proportion score over 50%, obtained a reduction of 70% in risk of disease progression or death from the illness with TiA *vs* A-P (HR 0.30; 95%CI: 0.15-0.61). This study appears to confirm the effectiveness of combining a dual inhibition of immunotherapeutic mechanisms and their potential benefit in clinical practice, although the final results and the study design of the phase III trial are still pending[7].

WHAT ARE THE BENEFITS OF IT COMBINATIONS TOGETHER WITH SHORT CT CYCLES?

Several preclinical studies have arrived at an interesting rationale to support CT-IT combinations. These combinations are an important challenge in NSCLC treatment, not only in advanced disease, but also in a neoadjuvant setting, where phase III studies such as CM816 have demonstrated a significant benefit of CT-IT pre-surgery compared to classical platinum based CT[19]. Although CT agents are regarded as immunosuppressants by directly inhibiting or killing effector cells, as well as indirectly by inducing anergy or immune paralysis[20], different combinations of CT and ICI have a proven synergistic effect. For example, CT aids in fast tumor regression and a reduction in tumor burden, and immune checkpoint blockade may prolong this effect, thus inducing a long-lasting anti-tumor response[21]. In addition, the non-overlapping toxicity profiles of IT and CT render them good candidates for combination strategies. On the hand, preclinical and clinical investigations have revealed that CT could enhance the efficacy of IT through various mechanisms. CT can induce immunogenic cell death and increase tumor antigen presentation and immune effector infiltration in tumors. Moreover, CT can modulate the immune microenvironment through mechanisms such as increasing cancer cell immunogenicity, enhancing the cytotoxicity of T cells and NK cells, and fostering the accumulation of IFN-γ and tumor necrosis factor (TNF)-α. The latest data show that CT also reduces the number of tumor immune suppressive cells like T regulatory cells, macrophages and neutrophils, and increases the ratio of cytotoxic T-cell/T regulatory cells and PD-L1 expression on tumor cells[22].

Specifically, platinum agents have been shown to partly exert their anti-tumor effect *via* modulation of the immune system. They attenuate STAT6 signaling by blocking STAT6 phosphorylation, resulting in a downregulation of PD-L2 on DCs and tumor cells. This triggers increased tumor cell recognition by T lymphocytes[23]. In a study of NSCLC cell lines, cisplatin upregulated the MHC class I chain-related molecule A and B expression, and led to enhanced NK cell-mediated antitumor effect[24]. Other mechanisms by which platinum agents stimulate the immune system include increasing human leukocyte 1 gene complex expression encoding MHC-I, which is associated with cytotoxic T-cell function[25].

As mentioned in previous sections, a number of clinical trials, such as the one described above, have focused on the administration of combinations of IT in first-line therapy. Overall, it is found that an unblocking of checkpoints of antitumor response

(PD-L1/CTLA-4), using combinations of checkpoint inhibitors, has proved more effective than CT alone or IT in monotherapy. The CM 227 trial, one of the first phase III studies that compared NI *vs* CT, reported a greater OS in the former treatment, regardless of PD-L1 expression[3].

In spite of the overall positivity of the CM 227 trial, an initial crossing of the curves takes place in the first 3-6 mo of follow up, both for the entire study population and also in patients with PDL-1 > 1%. This means that CT would be superior to IT in a profile of patients with more aggressive disease, who would potentially be more chemosensitive.

Another parallel study to CM227 in the PDL-1 > 25% population, also mentioned previously, is the MYSTIC study[4], which compares DT *vs* D *vs* CT. However, the results of this study were more disappointing, reporting no greater superiority for the IT doublet *vs* CT, although DT was more effective in the subgroup with TMB > 20. The CITYSCAPE study positions a new combination of IT (Ti-A) as another option in first-line therapy, and once again PD-L1 is a good predictor correlated with a greater benefit in favor of the combination.

Therefore, in spite of being a new option in first-line, d-IT was no more beneficial than its comparators in any of the studies analyzed, and which have been described here.

Subsequent studies, which aimed at improving the efficacy data of these schemes, incorporated CT regimens of different durations in an attempt to maximize the benefits of these new combinations.

With the goal of compensating for this initial deficit in OS in patients in the CM227 study, the CM9LA study added two cycles of platinum-based CT to the NI combination and achieved a greater benefit in OS for all the subgroups *vs* treatment with CT alone. However, as also reported previously in other studies on IT, no benefit in OS was found for patient smokers or for those aged over 75[5].

Another study that adds CT to the IT doublet is the aforementioned CCTG BR.34 study. This was designed with the same goal, in other words, to try to obtain better outcomes than the MYSTIC study[6]. In this case, efficacy data after a 16-month follow up found no differences in OS (with a non-significant favorable tendency in the PD-L1 > 50% group). However, differences were found in the response rates (28% *vs* 14%) and in PFS 7.7 *vs* 3.2 in favor of the combination with CT. A longer follow-up would probably produce more mature conclusions about the benefits of the IT doublet-CT combination *vs* the comparator with single IT-CT. In this case, in contrast to the MYSTIC study, there are no data to support a benefit in patients with a high TMB, although, once again, the high expression of PD-L1 would seem to predict a greater benefit.

In the debate about combinations of IT and CT, there is some controversy about the number of platinum cycles to use. Hence, the CM9LA study used 2 cycles while in the CCTG BR34 study, 4 cycles were administered. Although there are no data to attribute the difference in results to the number of CT cycles given, it is interesting that in the study with more CT cycles, in which better results would be expected, the data were, in fact, negative. It is, therefore, unclear whether the 2 first cycles are insufficient to compensate for the initial fall in the survival curve due to patients with an early sensitivity to IT.

On the other hand, there is no doubt about the rise in platinum-based toxicity of the longer CT schemes. Therefore, prospective studies may be necessary to determine whether reduced CT schemes, with greater tolerability, in combination with the IT doublet could be at least as effective as standard 4-cycle schemes. Another important aspect to consider about these new combinations concerns the choice of the best biomarker to predict benefits with the IT combination. Compared with PD-L1 expression, in the MYSTIC study higher levels of TMB predicted a greater benefit of the combo-IT. However, these results were not reproduced in other studies that analyzed this marker. For combinations of CT with anti-TIGIT/anti-PD-L1, data have not yet been published.

All these studies have only a short follow up and the results of longer follow up periods are required before we can confirm potential benefits for these new combinations of different and complementary therapeutic strategies.

TOXICITY

The toxicity of combinations with IT, whether this corresponds to d-IT or IT-CT, is highly variable, with cytostatic toxicity in addition to immune-mediated adverse

effects. The rates of grade 3-4 toxicities for the different studies on the d- IT combination are presented in Table 3.

Combinations of IT with the NI scheme in the CM227 trial[3], and with DT in the MYSTIC trial[4], show global rates of side effects of 76.7% and 60.1%, respectively; and of 54.2% in arm D. In both studies, higher rates were obtained for the control arm of CT, of 81.9% in CM227 and 83% in MYSTIC. For toxicity rates of G3 or higher, rates of 32.8% were recorded for the NI combination, 22.9% for the DT combination, 14.9% for D in monotherapy, and 33.8% and 36% in the CT arm, thus confirming in both studies that CT was more toxic than IT. Side effects of G3 or higher resulted in interruption of treatment in 9.4%-12.3% in the combination with the IT doublet, in 3.4%-4.9% in the CT arm and in 4.3% in arm D in monotherapy. As expected, the most frequent toxicities of CT were classical toxicities, such as pancytopenia, especially anemia and G3 neutropenia in 10%, and other common effects such as vomiting and asthenia. Immune-mediated adverse events were reported in 13.6% of arm D in monotherapy and in 28.3% with the DT combination. The most frequent immune-mediated toxicities corresponded to cutaneous reactions (30%-35%) and endocrine events (23%-25%). As published previously in other trials and studies, toxicity was also proportional to the level of PD-L1 expression. In CM227, patients with a level of expression of PD-L1 < 1%, presented less treatment-related G 3-4 toxicity (27.0 in PD-1 < 1% *vs* 32% general population).

As expected, combinations of IT-CT were also associated with an increased toxicity. In the CM 9LA trial[3], the global rate of adverse effects of G3 or higher was 47% in the experimental arm with NI-CT *vs* 38% in the control arm with CT, and slightly higher in the arms with CT of CM227[3] and MYSTIC[4]. In the CCTG BR.34 study[6], the combination of DT-CT presented side effects of G3 or higher in 82%, and in the control arm with DT alone of 70%, with rates of 22.9% reported in the MYSTIC study[4]. It is noteworthy that this toxicity is also higher, if results are extrapolated, than the toxicities of studies using the CT-IT combination such as Keynote 189, where a G3 toxicity of 67.2% was recorded[26].

The most common treatment-related serious adverse events of any grade were more frequent in the experimental group. These included diarrhea, febrile neutropenia, thrombocytopenia and anemia, with rates of 2%-3%. Treatment-related adverse events of severity G 3-4 that led to interruption of treatment were reported in 16%, and were higher than those reported for IT combinations in CM227[3], MYSTIC[4] and Keynote 189[26].

In studies of the doublet IT-CT, the most frequent treatment-related adverse events of any grade, which are commonly associated with CT, were anemia (23%-38%), neutropenia (10%-17%) and thrombocytopenia (5%-10%) in CM 9LA[5]. Grade 3-4 adverse events in the CCTG BR.34 study corresponded to neutropenia (7%-9%), anemia (6%-14%), diarrhea (1%-4%), elevated lipase levels (1%-6%) and febrile neutropenia (3%-4%). In the CM9LA study[3], the commonest immune-mediated adverse events of level G 3-4 were gastrointestinal (6%), cutaneous (4%) and hepatic (4%). Most of these were successfully resolved, with some patients requiring immune modulating medication, including corticosteroids and TNF antagonists. In the CCTG BR 34 trial[4], the incidence of adverse events related to the immune system was similar in all arms (colitis 11%, pneumonitis 6%, endocrinopathy 21%).

The toxicity of the combination of A and Ti was not negligible, (CITYSCAPE)[7], with global rates of side effects of 80.6% *vs* 72%, respectively, and G3 toxicity rates of 19.1% *vs* 14.9%, and rates of interruption of treatment of 10.3% *vs* 7.5% for the control group. Once again, in the experimental arm the most frequent immune-mediated toxicity effects were skin rashes, perfusion reaction, pancreatitis, thyroid alterations and colitis.

The toxicity of the IT combinations is, therefore, greater than that of studies of single IT, at the expense of more immune-mediated effects, as to be expected. Similarly, toxicity increases when CT is added to these IT doublet schemes because of the toxicity associated with platinum regimens. Indirect comparisons of the more classical schemes of m-IT-CT *vs* d-IT-CT show greater toxicity in the latter, directly proportional to the number of CT cycles. Hence, in the decision-support algorithm to select the best first-line scheme in our patients, it is essential to take into account the toxicity profiles of each alternative treatment.

CONCLUSION

The combination of different checkpoint inhibitors, or checkpoint inhibitors combined

Table 3 Summary of the main toxicities in each of the studies on immunotherapy combinations or immunotherapy ± chemotherapy

Phase II-III studies	G3-4 toxicity (%)		Treatment discontinuation (%)	
	Exp arm	Control arm	Exp arm	Control arm
CM 227	32.8 NI	33.8 CT	9.4 NI	3.4 CT
MYSTIC	22.9 DT	36 CT	12.3 DT	4.9 CT
CM9LA	47 NI-CT	38 CT	16 NI-CT	9 CT
CCTG-BG-34	82 DT-CT	70 DT	NA	NA
CITYSCAPE	19 ATir	14 PA	10.3 ATir	7.5 PlA
KEYNOTE-189	67 P-CT	65.8 CT	13.8 P-CT	7.9 CT

NI: Nivolumab + ipilimumab; DT: Durvalumab+ tremelimumab; CT: Chemotherapy; Tir: Tirogolumab; A: Atezolizumab; PL: Placebo; P-CT: Pembrolizumab + chemotherapy; NA: Not available.

with other immune modulating strategies, such as TIGIT inhibition, in the context of first-line therapy in advanced NSCLC produces superior outcomes to classical CT regimens. The addition of CT (of 2 to 4 cycles) to this IT doublet is a new strategy to rescue patients who could initially present a detrimental clinical course after first receiving the IT doublet. It is necessary to continue researching to find more accurate predictive biomarkers of response to optimize the selection of patients who should receive these types of therapeutic strategies.

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MINIREVIEWS

Role of liver transplantation in the management of colorectal liver metastases: Challenges and opportunities

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Abstract

The liver is the most common site of colorectal cancer metastasis. Complete resection of the metastatic tumor is currently the only treatment modality available with a potential for cure. However, only 20% of colorectal liver metastases (CRLM) are considered resectable at the time of presentation. Liver transplantation (LT) has been proposed as an alternative oncologic treatment for patients with unresectable CRLM. This review summarizes the published experiences of LT in the setting of unresectable CRLM from the previous decades and discusses the challenges and future horizons in the field. Contemporary experiences that come mostly from countries with broader access to liver grafts are also explored and their promising findings in terms of overall survival (OS) and disease-free survival (DFS) are outlined along with their study design and methods. The rationale of establishing specific patient selection criteria and the dilemmas around immunosuppressive regimens in patients undergoing LT for CRLM are also highlighted. Additionally, this review describes the findings of studies comparing LT *vs* chemotherapy alone and LT *vs* portal vein embolization plus resection for CRLM in terms of OS and DFS. Last but not least, we present current perspectives and ongoing prospective trials that try to elucidate the role of LT for CRLM.

Key Words: Colorectal cancer; Colorectal liver metastases; Liver transplantation;

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Core Tip: Despite the discouraging results of the previous decades, reports from the recent era showed promising results and reemerged the idea of liver transplantation (LT) for colorectal liver metastases (CRLM). Documentation of patient selection criteria and stronger evidence from ongoing prospective trials may reinforce the implementation of LT as an oncologic treatment for CRLM.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancer entities worldwide, ranking third in terms of incidence, and second in terms of cancer-related death[1]. The overall survival of the patients with CRC depends primarily on cancer staging[2-4]. The liver is the most common site of CRC metastasis, mainly due to its anatomical association with the portal circulation[3]. Published data indicate that approximately 20% of patients with CRC present with concomitant liver metastasis at the first medical consultation, while another 50% develops liver metastasis within the first 3 years after primary tumor diagnosis[2,3,5,6]. The life expectancy of patients with colorectal liver metastasis (CRLM) who do not receive any type of treatment ranges from 12 to 15 mo, and the 5-year survival is less than 5%[2]. Implementation of chemotherapy as the only treatment modality for CRLM results in a median patient survival of approximately 25 mo[7]. Complete tumor resection is currently the only potentially curative treatment with a 5-year and 10-year overall survival (OS) of 38% and 26%, respectively[8]. Yet, only 20% of patients present with a hepatic lesion that can be managed surgically with a curative intent[9-11]. Additionally, what constitutes a surgically resectable CRLM is a matter of debate among surgeons[12,13]. Recent advances in surgical techniques[14] combined with the emergence of newer chemotherapeutic drugs[15,16] have increased the proportion of CRLM amenable to resection. Unfortunately, disease recurrence is still reported in 40%-70% of patients within the first 3 years after surgical excision[17,18] due to the presence of micro-metastatic disease, resulting in a median OS of 10-38 mo for patients relegated to palliative chemotherapy[19,20].

This has led to the consideration of liver transplantation (LT) as an oncologic treatment for patients with CRLM isolated to the liver[9,21]. The aim of this review is to delineate the rationale and outcomes of LT in the setting of unresectable CRLM, and to outline the potential benefits, future perspectives, and ethical dilemmas about this treatment modality.

EARLY EXPERIENCE

LT was historically first attempted in patients with malignant liver tumors (including patients with CRLM)[22]. However, poor survival and high recurrence rates quickly led to restriction of LT utilization to early-stage hepatocellular carcinoma (HCC). The experience gained through the years along with advances in surgical technique and neoadjuvant modalities have broadened the spectrum of malignant indications for LT including advanced HCC, hilar cholangiocarcinoma, as well as metastatic liver tumors (*e.g.*, neuroendocrine metastasis)[23]. This motivated some groups in 1990s to re-assess the role of LT for unresectable CRLM.

The first report of LT for CRLM was from Medical University of Vienna, Austria [24]. Mühlbacher *et al*[24] reported a series of 25 patients who underwent LT for CRLM between 1982-1994 (all patients had lymph node negative disease). In this study, the 1-,

3-, and 5-year post-LT OS was 76% (19/25), 32% (8/25), and 12% (3/25), respectively [19,24]. It should be acknowledged, that after retrospective histological examination of the excisional specimens, lymph node micro-metastases were observed in 15 out of 21 patients who were initially classified as having negative lymph node status. This finding was associated with a decreased post-LT median survival of 28 mo compared to a median survival of 118 mo in patients without micro-metastases[24-26]. Another early experience was published in 1991 by Penn *et al*[27] from University of Cincinnati reporting on 10 patients undergoing LT for CRLM (eight of them due to unresectable tumor and two of them due to chemotherapy adverse effects) with a 70% recurrence rate. Additionally, Pichlmayr *et al*[28] published another series of patients undergoing LT in Germany during 1972-1995, and amongst the reported cases there were 4 patients who underwent LT for CRLM. Two of these patients died in the early post-operative period (one due to acute graft rejection), while the other two patients died from disease recurrence at 11 mo and 33 mo follow-up[28]. The discouraging results from these studies in addition to the lack of standardized criteria for patient selection led to CRLM being established as a formal contraindication for LT over the next decades.

RECENT ERA

The broader access to deceased donor liver grafts in Norway led a group from Oslo University Hospital to investigate the outcomes of well-selected LT candidates with unresectable liver-only CRLM[9,21,29,30]. The first prospective study (SECA-I) was published in 2013 and included 21 patients who had undergone LT from 2006 to 2011 [30]. Inclusion criteria were total resection of the primary tumor, ECOG score 0 or 1, at least 6 wk of neoadjuvant chemotherapy, and absence of extrahepatic disease[30]. Liver resection prior to LT had been performed in 3 patients. The median follow-up time was 27 (range, 8-60) months and the 1-, 3-, and 5-year OS was 95%, 68%, and 60%, respectively. All patients received sirolimus for immunosuppression and none of them received adjuvant chemotherapy. Disease free survival (DFS) was 35% at 1 year[30]. Another publication from the same group reported a total of 19 recurrences in 21 patients (16 were single-site and 3 were multiple-sites at first presentation)[29]. Thirteen of the 16 recurrences were isolated to the lung and patients with isolated pulmonary metastases had a 5-year survival of 72% after recurrence was diagnosed [29]. Notably, there was no isolated hepatic recurrence at initial presentation[29]. However, seven patients developed metastasis to the transplanted liver on subsequent follow-up and six out of those seven patients eventually died from metastatic disease.

Although the results from SECA-I were encouraging, the high recurrence rates led to more stringent candidate selection criteria in SECA-II. SECA-II included 15 patients who had undergone LT for unresectable liver-only CRLM from 2012 to 2016[9]. Similar to the SECA-I trial, all patients received sirolimus for immunosuppression and none of them received adjuvant chemotherapy. The stricter selection criteria required that isolated liver-only CRLM was confirmed by 18F-fluorodeoxyglucose (18F-FDG) positron emission tomography (PET)/computed tomography (CT) scan and patients had more than one year time span from diagnosis of CRC to LT. Additionally, at least 10% response on chemotherapy (according to RECIST-criteria) was a prerequisite for inclusion in the SECA-II study[9]. Resection prior to LT was performed in 4 patients. Median follow up was 36 mo. Compared to SECA-I, the more restrictive selection criteria led to improved 1-, 3-, and 5-year OS of 100%, 83%, and 83%, respectively. However, median DFS remained low at 13.7 mo. Overall, 8 patients were reported to have disease recurrence after LT and 6/8 presented with isolated pulmonary metastasis. On follow-up, 13 patients were alive, and 2 patients died 26 mo after LT due to disease recurrence. The main limitations of this study were the small sample and the relative short follow-up time, but the encouraging results drove the investigators from Oslo to conduct an additional enrollment to the SECA-II study using grafts from extended criteria donors[21]. This study (D-arm of SECA-II) included both patients with synchronous CRLM (within 1 year of primary colorectal tumor diagnosis) and those with concomitant resectable pulmonary metastases or with previously resected pulmonary metastases[21]. Moreover, the investigators did not consider response to chemotherapy as a prerequisite for recruitment[9,21]. Ultimately, 10 patients were included between 2014-2018. The median follow up was 23 mo, the median OS was 18 mo, and the median DFS was 4 mo. Disease recurrence was noted in 8/10 patients with isolated pulmonary metastasis seen in six patients. Overall, five patients were still alive on follow-up with two of them having no relapse at 23 mo and

26 mo after LT[21]. These outcomes established that LT could be considered in patients with unresectable liver-only CRLM only under strict selection criteria.

An international, multicenter, retrospective study of 12 patients was published by Toso *et al*[31] in 2017. Eleven of the patients had received chemotherapy prior to LT. The median follow-up time was 26 mo and the 1-, 3-, and 5-year OS was 83%, 62%, and 50%, respectively, while the 1-, 3-, and 5-year DFS was 56%, 38%, and 38%, respectively. Disease recurrence was noted in six patients with five of them presenting with pulmonary metastasis, while 5 out of the 11 patients were reported to be alive and without evidence of relapse at the end of the follow-up[31]. However, due to the nature of the study patients were not selected according to homogeneous criteria and they were not managed with the same interventions. Despite the limitations, this report demonstrated that LT for CRLM can provide a survival benefit in carefully selected patients, but additional refinement is necessary prior to the broader application of LT as an oncologic treatment for CRLM.

A recent worldwide systematic review and pooled analysis of 110 patients undergoing LT for CRLM reported that the 1-, 3-, and 5-year OS rates were 88.1%, 58.4%, and 50.5%, respectively[32].

Study characteristics and findings for the early experience and recent studies on LT for CRLM are shown in Table 1.

RISK STRATIFICATION CRITERIA

Similar to prior reports establishing specific selection criteria for other liver malignancies (Milan criteria for HCC and Mayo Clinic criteria for hilar cholangiocarcinoma)[33,34] the SECA studies introduced the Oslo score which was used a surrogate marker for favorable prognosis[9,21,30]. One point was assigned for each of the following characteristics: (1) Lesion larger than 5.5 cm; (2) Pre-LT plasma carcinoembryonic antigen (CEA) level above 80 μg/L; (3) Time from primary tumor resection to LT less than 24 mo; and (4) Disease progression while on pre-LT chemotherapy. Each of these factors was significantly associated with poorer OS and the five patients who possessed all four factors comprised five of the six mortalities in the SECA-I trial[30]. Risk stratification was also done utilizing the Fong Clinical Risk Score (FCRS)[35], in which one point was given for the following: (1) Synchronous CRLM (less than 12 mo from diagnosis); (2) Primary tumor with positive lymph nodes; (3) More than one lesion; (4) Tumor larger than 5 cm; and (5) CEA level higher than 200 μg/L. FCRS of 1-2 at the time of diagnosis was associated with significantly increased DFS compared to FCRS of 3-4[9].

The importance of stricter patient selection was also highlighted by the differences in OS and DFS between SECA-I and SECA-II studies, where 5-year OS was 60% and the 1-year DFS was 35% for SECA-I, while the 5-year OS was 83% and the 3-year DFS was 35% for SECA-II[9,29]. Smedman *et al*[21] attributed the poorer outcomes in terms of survival and disease recurrence of the D-arm of SECA-II in the significantly higher Oslo and FCRS scores of the patients compared to the patients of SECA-I and primary SECA-II trials[9,21,29,30]. Therefore, it is apparent that strict patient selection criteria and risk stratification are essential for the broader adoption of LT as a life prolonging oncologic treatment for liver-only CRLM.

IMMUNOSUPPRESSION

Immunosuppression is a controversial topic regarding LT for metastatic diseases in terms of achieving a balance between the risk of graft rejection and the risk of disease recurrence[19,32]. That is because attenuation of the native immune response from immunosuppression is essential to prevent graft rejection, however, it may contribute to unfavorable post-LT outcomes in patients with disseminated malignant disease, as it could facilitate disease recurrence. A study that assessed the impact of sirolimus post-LT for HCC, documented that immunosuppression improved the outcomes in the first few years post-LT and had no effect in DFS or OS beyond 5 years post-LT[36]. Notably, sirolimus was the immunosuppressive regimen used in the SECA trials[9,21,29,30]. Data from a study that compared the growth of pulmonary metastasis in patients enrolled in the SECA trials *vs* patients with CRC and lung metastasis who did not receive immunosuppression, reported that there was significant difference between the two groups in terms of the time needed to double tumors' diameter and volume[37]. Moreover, the same study reported that there was no association between

Table 1 Study characteristics and findings

A. Early experience

Author, Yr	Study period	Number of patients	Clinical outcomes
Mühlbacher *et al*[24], 1991	1982-1994	25	1-yr OS: 76%, 3-yr OS: 32%, 5-yr OS: 12%
Penn *et al*[27], 1991	N/A	10	70% recurrence rate
Pichlmayr *et al*[28], 1997	1972-1995	4	2 post-operative mortalities, 2 late mortalities due to recurrence

B. Recent era

Author, Yr	Study period	Number of patients	Follow-up	1-yr OS, %	3-yr OS, %	5-yr OS, %	DFS
Hagness *et al*[30], 2013	2006-2011	21	27 mo	95	68	60	35% at 1st year
Dueland *et al*[41], 2020	2012-2016	15	36 mo	100	83	83	13.7 mo
Smedman *et al*[21], 2020	2014-2018	10	23 mo	N/A	N/A	N/A	4 mo
Toso *et al*[31], 2017	1995-2015	12	26 mo	83	62	50	56% at 1st year

All values reported for continuous variables are expressed in median. OS: Overall survival; DFS: Disease free survival, N/A: Not available.

sirolimus plasma levels and DFS or growth of pulmonary metastases[37]. However, the current level of evidence is relatively low, and future high-quality studies are required to draw solid conclusions for immunosuppressive therapies after LT for CRLM.

LIVER TRANSPLANTATION VERSUS CHEMOTHERAPY

In 2015, Dueland *et al*[38] published a study aiming to outline the differences in OS of patients with CRC and nonresectable CRLM treated by LT or chemotherapy. The investigators compared the SECA-I study with the NORDIC VII study, which was a multicenter randomized three-arm trial investigating the efficacy of cetuximab added to bolus fluorouracil/folinic acid and oxaliplatin[39]. The 21 patients from SECA-I study were compared with the 47 patients from the NORDIC VII study, in terms of DFS and OS. DFS was 8 to 10 mo in both groups[38]. However, the 5-year OS was 56% in patients undergoing LT compared to 9% in patients receiving chemotherapy[38]. The authors attributed this difference to the pattern of disease recurrence. While small and slow growing lung metastases were the most common recurrence pattern in the LT group, patients in the chemotherapy group presented with progression of the nonresectable CRLM, which has a less favorable prognosis[38].

LIVER TRANSPLANTATION VERSUS PORTAL VEIN EMBOLIZATION PLUS LIVER RESECTION

Emerging surgical advances have been proposed to increase the pool of patients with CRLM that can be subjected to liver resection. Implementation of portal vein embolization (PVE) could render initially nonresectable CRLM amenable to resection [40]. Dueland *et al*[41] compared 50 patients enrolled to SECA studies with a matched group of 53 intention-to-treat patients who have undergone PVE to expand the future liver remnant (FLR) and were planned to undergo liver resection (15 patients did not proceed to liver resection due to inadequate FLR or disease progression). Although the data for the whole LT cohort are not presented to clearly appreciate differences compared to the PVE cohort, the authors mentioned that the two groups had similar selection criteria. Additionally, patients were subclassified in two subgroups; the high tumor load (HTL) group was defined as patients having ≥ 9 metastatic tumors or largest tumor diameter ≥ 5.5 cm, while patients with CRLM below the aforementioned limits were included in the low tumor load (LTL) group[41]. The 5-year OS for patients with HTL was 33.4% in the LT arm (n = 29) compared to 6.7% in the PVE arm (n = 15) of the study without any between-group differences regarding tumor burden score. The 5-year OS for patients with LTL was 72.4% in the LT arm (n = 21) compared to 53.1% in the PVE arm (n = 30), while the tumor burden score was significantly higher

in the LT arm. Accounting that there are no randomized controlled trials comparing LT to PVE plus resection in patients with extensive liver-only CRLM, as well as the fact that these two modalities may not necessarily be applicable to the same pool of patients, this study provides some evidence that LT has promising future perspectives in the field of oncologic treatments for CRLM.

CURRENT AND FUTURE PERSPECTIVES

The International Liver Transplantation Society Transplant Oncology Consensus Conference recommendations, based on the findings of SECA trials, suggested that LT could be implemented in patients with unresectable CRLM with only liver involvement and with a maximum tumor diameter ≤ 5.5 cm, pre-LT CEA ≤ 80 μg/L, response to pre-LT chemotherapy, and time interval from diagnosis to LT ≥ 1 years [42]. However, worldwide liver graft scarcity poses an ethical dilemma which is summarized as follows: How will the distribution of existing grafts to patients with CRLM impact patients with imperative need for a graft? In the United States, the Model for End-Stage Liver Disease score is used to prioritize patients for LT based on severity of liver derangements. However, patients with non-resectable CRLM have no portal hypertension or liver disease and thus are handicapped for access to deceased donors. Such patients could be good candidates for living donor liver transplantation (LDLT). Consequently, considering a long-term OS in the order of 60%, LDLT could offer a very good therapeutic alternative to this group of patients without jeopardizing the cadaveric donor pool.

Several trials attempting to elucidate the role of LT in CRLM are currently ongoing. In addition to the SECA-I (NCT00294827 - active, not recruiting: estimated study completion date May 2023) and SECA-II (NCT01479608 - active and recruiting: estimated study completion date December 2027), the Oslo group is also working on the SECA-III study, which aims to assess the efficacy of LT *vs* other therapies (chemotherapy and surgical resection) with a primary outcome of 2-year OS, and the RAPID (NCT02215889) trial, which aims to evaluate the outcomes of recipient left lateral segmentectomy and implantation of donor segments 2 and 3 followed by removal of the remaining recipient liver segments (second stage hepatectomy) at 4 wk post-LT. The LIVERT(W)OHEAL trial (NCT03488953) will evaluate the outcomes of LDLT in both the donors and the recipients. The largest ongoing trial, estimated to eventually enroll approximately 90 patients, is the TRANSMET (NCT02597348) phase III randomized controlled trial and will evaluate the 3-year OS and disease recurrence or progression in patients with CRLM undergoing LT plus chemotherapy *vs* chemotherapy only. Finally, a trial conducted by the Toronto group (NCT02864485), the COLT trial (NCT03803436), the TRIPLETE trial (NCT03231722), and the Swedish SOULMATE trial (NCT04161092) are also ongoing trials that investigate the utilization of LT as an oncologic treatment for CRLM.

Several other perspectives on the assessment for candidacy should also be incorporated into future studies. FDG is widely used to stage and monitor treatment response in metastatic CRC and the use of PET/CT scan to stage patients, as well as to assess response to therapy has been raised as a parameter of interest. Mutational profiling of CRC has been shown to have an impact on patient outcomes[43], and thus the role of selecting patients for LT based on mutational profiling will need to be addressed. Finally, the use of neoadjuvant radiotherapy to the native liver prior to LT to reduce intraoperative shedding of tumor cells during hepatectomy is also under consideration.

CONCLUSION

The SECA studies from Oslo have demonstrated promising results in prolonging survival with the use of LT as an oncologic treatment for carefully selected patients with unresectable liver-only CRLM. Further evidence is currently awaited from ongoing prospective trials in order to better define the role of LT for unresectable CRLM. The addition of unresectable CRLM as an indication for LT represents a paradigm shift and further confirms versatility of the emerging field of transplant oncology.

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ORIGINAL ARTICLE

Basic Study

Increased tensin 4 expression is related to the histological type of gastric cancer

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Abstract

BACKGROUND

Gastric cancer (GC) is one of the most common malignant tumors worldwide. Tensin 4 (TNS4) is an adhesive protein belonging to the tensin family. This protein is located in focal adhesion sites. The *TNS4* gene is considered an oncogene in numerous cancers. This protein plays an important role in adhesion, migration and proliferation of cells.

AIM

To evaluate expression of TNS4 protein in GC tissues and analysis of the clinical and histopathological parameters as well as the overall survival rate of patients.

METHODS

The expression of TNS4 was assessed in 89 patients using immunohistochemistry.

RESULTS

Positive expression of TNS4 was observed in 49 of 89 patients (55.06%). Higher TNS4 expression was more common in GC tumors with a diameter ≥ 5 cm (P = 0.040). We demonstrated that an increase in TNS4 expression was more frequent in tumors of the histological type without mucinous components than in tumors from mucosal cancers (P = 0.023). Furthermore, TNS4 expression was higher in moderately differentiated tumors than in poorly differentiated and non-differentiated tumors (P = 0.002). Increased TNS4 expression was also noted in the intestinal type of GC according to Lauren's classification (P = 0.020). No statistically significant correlation was found between the expression of TNS4 and the

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overall survival rate of patients.

CONCLUSION
TNS4 expression was significantly higher in tumors with a diameter ≥ 5 cm of the moderately differentiated intestinal type (according to Lauren's classification) of GC without a mucinous component. Therefore, increased TNS4 expression is related to the histological type of GC with a better prognosis.

Key Words: Gastric cancer; Tensin 4; Adhesion proteins; Immunohistochemistry

Core Tip: Tensin 4 (TNS4) is an adhesive protein belonging to the tensin family that plays an important role in cell adhesion, migration, and proliferation. These processes are important in cancer development and may limit cancer cell growth and improve patient survival. By applying immunohistochemistry, we investigated TNS4 expression in gastric cancer (GC) tissues and discovered that TNS4 expression was significantly higher in tumors with a diameter greater than 5 cm, in tumors of the moderately differentiated intestinal type (according to Lauren's classification) and in GC without a mucinous component. We concluded that enhanced TNS4 expression was associated with the histological type of GC with a better prognosis.

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INTRODUCTION

In 2018, more than one million people were diagnosed with gastric cancer (GC), accounting for 5.7% of all malignant cancers worldwide; this cancer is ranked 5th in terms of incidence. Moreover, 8.2% of patients with GC died, which reveals the aggressive nature of this disease. GC was diagnosed twice as often in men than in women[1]. The development of this cancer is a multistage process, and there are numerous environmental and genetic factors that contribute to its progression, including infection with *Helicobacter pylori* (*H. pylori*) bacteria and Epstein-Barr virus, consumption of excess salt and alcohol or smoking[2]. An alarming phenomenon is the incidence of this type of cancer in young people under 40 years of age, most often as a result of a genetic predisposition. A previous report showed that 30% of patients under 30 had GC in their family history[3]. The diagnosis of this cancer at early stages of its progression is of key importance. Preoperative chemotherapy followed by surgical removal of the tumor mass in early cancer results in a 90% 5-year survival rate [4]. Therefore, it is crucial to search for molecular and biochemical changes typical of early stages of cancer development to inhibit tumor progression and increase the survival rate of patients with this type of cancer.

Tensin 4 (TNS4), also known as C-terminal tensin-like protein, belongs to the tensin family, which also includes tensins 1–3. The *TNS4* gene is located on the long arm of chromosome 17q12-21. The TNS4 protein has a molecular weight of 77 kDa. In terms of structure, TNS4 is composed of the Src homology 2 domain (SH2) and the phosphotyrosine-binding domain (PTB). The SH2 domain allows the TNS4 protein to participate in the transmission of intracellular signals, while the PTB domain binds to membrane integrator receptors. Unlike tensins 1–3, TNS4 does not have an actin-binding domain[5]. Intracellularly, TNS4 is located in focal adhesion sites, where it enables signaling between the extracellular matrix and the cell interior. From a physiological perspective, the expression of TNS4 has been detected in the prostate and placenta[6]. Pathologically increased TNS4 expression appears in cancerous tumors. *TNS4* was originally classified as a suppressor gene in prostate cancer[6], but as research on tensins progressed, its role as an oncogene was demonstrated in

colorectal, breast, colon, esophagus, and lung cancers and thymoma, where this protein was overexpressed[7-15]. TNS4 plays an important role in biological processes connected with carcinogenesis, such as proliferation, migration, cell adhesion and invasiveness[16].

The aim of our study was to evaluate the IHC expression of TNS4 protein in GC (stages I-IV) as well as to assess the relationship between protein expression and selected clinical and pathological parameters and the overall survival rate of patients.

MATERIALS AND METHODS

Study group

The study was conducted on a group of 89 patients diagnosed with GC who were treated surgically in the 2nd Clinical Department of General and Gastroenterological Surgery of the Medical University of Bialystok in the years 2005–2015. The tissue material was obtained from the archives of the Academic Center for Pathomorphological and Genetic-Molecular Diagnostics in Bialystok. The inclusion criterion was diagnosed adenocarcinoma at any stage of its progression; the exclusion criteria were diagnosed squamous cell carcinoma and other non-epithelial cancers, metastases of other cancers to the stomach and the lack of complete medical records. The normal mucous membrane was used as the control tissue. The research was approved by the Bioethics Committee of the Medical University of Bialystok, permission No. R-I-002/29/2019. The study was conducted in accordance with the World Medical Association Declaration of Helsinki for ethical principles for medical research involving human subjects. The characteristics of the study group are presented in Table 1.

Tissue preparations

Tissues collected during the operation were fixed in 4% buffered formalin and embedded in paraffin. Paraffin blocks were then cut on a microtome into approximately 4-μm-thick sections and stained with hematoxylin and eosin. A routine histopathological examination included the assessment of the histological type of cancer, malignancy grade (G), anatomoclinical stage (pT) and presence of lymph node metastases (pN), infiltration of blood and lymphatic vessels, perineural infiltration, peritumoral inflammation and degree of desmoplasia. Moreover, *H. pylori* infection was assessed by Giemsa staining. The following information was selected from the records with histopathological diagnosis: age and sex of patients, diameter and location of the tumor, presence of distant metastases and the type of cancer according to Lauren's classification.

Immunohistochemistry

Immunohistochemical (IHC) staining was performed on 89 GC tissues. Paraffin blocks were cut on a microtome into approximately 4-μm-thick sections and mounted onto silanized slides. The microscopic sections were incubated overnight at 60 °C and then deparaffinized in xylene solutions and hydrated in a series of alcohol solutions of decreasing concentration (2 × 99.9%, 96%, 70%). The next step was blocking endogenous peroxidase activity by using 3% hydrogen peroxide solution (10 min) as well as nonspecific antibody binding by means of horse serum (anti-mouse/rabbit serum produced in horse, Vector Laboratories, Germany) (20 min). In the following step, the sections were incubated with polyclonal anti-TNS4 antibody at a dilution of 1:75 (Biorbyt, orb186458 produced in rabbit) for 30 min at room temperature. The antibody binding sites were visualized with an ImmPress Universal Antibody Polymer Reagent kit (Vector Laboratories, Germany) and ImmPACT DAB chromogen (Vector Laboratories, Germany). Cell nuclei were stained with hematoxylin. The preparations were then dehydrated in a series of alcohol solutions of increasing concentration and overexposed in xylene solutions.

Validation of TNS4 expression detection

We particularly focused on obtaining reliable results of TNS4 IHC staining. To optimize the TNS4 staining procedure, we used positive and negative controls, and selected primary antibody dilutions (1:50, 1:75, 1:100, 1:200) and incubation times (30 min, 60 min, 120 min) were tested. For the control, antigen retrieval was also performed in buffers with pH = 6.0 and pH = 9.0.

Table 1 The characteristics of study group

Parameter	Number of cases
Age	
< 60	29 (32.58%)
≥ 60	60 (67.42%)
Gender	
Female	29 (32.58%)
Male	60 (67.42%)
Tumor diameter	
< 5 cm	18 (20.22%)
≥ 5 cm	71 (79.78%)
Tumor localization	
Upper 1/3	17 (19.10%)
Middle1/3	33 (37.08%)
Lower 1/3	16 (17.98%)
Whole stomach	23 (25.84%)
Histological type	
Adenocarcinoma	54 (60.67%)
Adenocarcinoma mucinosum	35 (39.33%)
Histological differentiation	
Moderately differentiated	25 (28.09%)
Poorly differentiated	35 (39.33%)
Non differentiated	29 (32.58%)
Depth of invasion	
T1	6 (6.74%)
T2	7 (7.87%)
T3	66 (74.16%)
T4	10 (11.23%)
Lymph node metastasis	
Absent	17 (19.10%)
Present	72 (80.90%)
Distant metastasis	
Absent	61 (68.54%)
Present	28 (31.46%)
Blood vessel infiltration	
Absent	48 (84.21%)
Present	9 (15.79%)
Lymphatic vessel infiltration	
Absent	21 (31.82%)
Present	45 (68.18%)
Perineural infiltration	
Absent	28 (34.15%)
Present	54 (65.85%)

Peritumoral inflammation	
Absent	42 (50.00%)
Present	42 (50.00%)
Desmoplasia	
Small	55 (66.27%)
Large	28 (33.73%)
Helicobacter pylori infection	
Absent	61 (73.49%)
Present	22 (26.51%)
Lauren's classification	
Intestinal	44 (55.00%)
Diffuse	36 (45.00%)

Microscopic evaluation

The preparations were examined under an Olympus BX41 light microscope by two independent pathomorphologists. TNS4 protein expression was evaluated under 400× magnification in 10 representative fields of view. In each field of view, 100 or more cancer cells were assessed. Protein expression was observed in both the cell membrane and the cytoplasm. The presence of TNS4 protein in ≥ 20% of neoplastic cells was considered positive expression.

Statistical analysis

The comparison of TNS4 expression with the selected clinicopathological parameters was carried out by the Mann-Whitney U test for two groups and the Kruskal-Wallis test for 3 or more groups. Dunn's multiple comparison post hoc tests were conducted for the Kruskal-Wallis test. A value of $P < 0.05$ was considered statistically significant. The overall survival analysis was based on the Kaplan-Meier test. The program Statistica 13 (Statsoft, Krakow, Poland) was used for the analysis. Missing data were removed in pairs.

RESULTS

TNS4 expression in GC samples

TNS4 protein expression was tested immunohistochemically in 89 GC samples and 20 normal stomach tissues. Statistical analysis demonstrated that positive TNS4 expression in cancer cells occurred in 49 of 89 patients (55%). In neoplastic cells, the expression of TNS4 was observed in the cell membrane and cytoplasm (Figure 1)

Analysis of the correlation of TNS4 expression with clinical and pathological parameters of GC

The statistical analysis did not show any significant relationships between TNS4 protein expression and the age and sex of patients, tumor location, anatomoclinical stage–pT, presence of lymph node and distant metastases, blood and lymphatic vessel infiltration, perineural infiltration, peritumoral inflammation, desmoplasia or *H. pylori* infection. However, a significant correlation was found between TNS4 expression and tumor diameter. Tumors with a diameter of < 5 cm were present in 20.22% of the patients, while tumors with a diameter of ≥ 5 cm were found in 79.78% of the patients. Positive expression of the TNS4 protein was more frequent in gastric tumors with a diameter of ≥ 5 cm (60.56% of the patients with positive expression) than in tumors < 5 cm in diameter (33.33% of the patients) ($P = 0.040$) (Figure 2A). Furthermore, a significant correlation with the histological type of tumor was demonstrated. Cancers without mucinous components were diagnosed in 60.67% of the patients, and mucosal tumors were diagnosed in 39.33% of the patients. A positive reaction to the TNS4 protein was considerably more frequent in the histological type without a mucinous component (64.81% of patients) than in mucinous adenocarcinomas (40.00% of patients) ($P = 0.023$) (Figure 2B). Statistical analysis also showed a significant

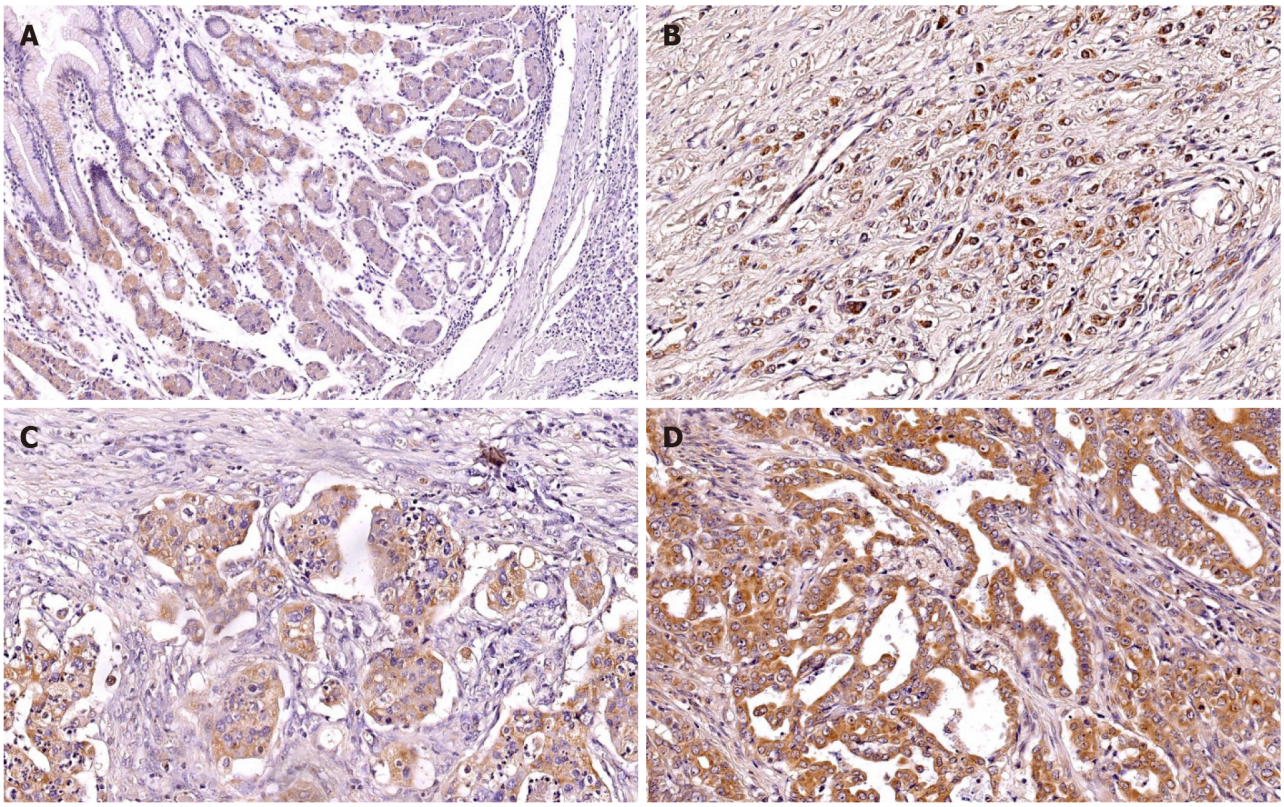

Figure 1 Tensin 4 expression in normal gastric mucosa. A: 100× magnification and gastric cancer samples; B and C: Poorly differentiated gastric cancer (GC); D: Moderately differentiated GC (magnification 200×).

correlation with the malignancy grade. In the study group, there were 28.09% moderately differentiated, 39.32% poorly differentiated and 32.59% non-differentiated tumors. The TNS4 protein occurred much more frequently in moderately differentiated cancers (80.00% of the patients) than in poorly differentiated (57.14%) and non-differentiated (31.03%) tumors (P = 0.002) (Figure 2C). It was also proven that TNS4 protein expression correlates with the type of tumor according to Lauren's classification. The intestinal type was present in 55% of the patients, while 45% of the patients suffered from the diffuse type of GC. The presence of TNS4 was more frequently observed in intestinal-type tumors (70.45%) than in diffuse-type tumors (44.44%) (P = 0.020) (Figure 2D).

Table 2 presents a summary of the correlation between clinicopathological parameters and TNS4 protein expression found in our study.

Analysis of the correlation of TNS4 protein expression with the overall survival rate

Statistical analysis did not reveal a significant correlation between TNS4 protein expression and overall patient survival (P = 0.25) (Figure 3).

DISCUSSION

In our study, we performed an IHC assessment of the expression of the TNS4 protein in GC patients diagnosed with stage I to IV disease. We observed that TNS4 expression was higher in tumor cells than in normal tissue, which is consistent with the results presented by Qi *et al*[17]. Sawazaki *et al*[18] evaluated the clinical significance of *TNS4* mRNA expression in GC and demonstrated that its expression is significantly higher in tumor tissue, as confirmed by Sakashita *et al*[19]. Based on these observations, it can be concluded that changes in the expression of the *TNS4* gene occur in tumor cells compared to normal gastric mucosa cells. This change is reflected in increased expression of TNS4 protein in GC.

In our research, we demonstrated that positive expression of TNS4 protein occurs twice as often in GC tumors with a diameter of ≥ 5 cm than in tumors with a diameter smaller than 5 cm. Similar observations were reported by other authors who noted that TNS4 protein expression increases with the size of breast tumors and hepatocellular

Table 2 Correlation between clinical pathological parameters and tensin 4 protein expression

Parameter	Expression of tensin 4 protein		[a]*P* value
	Negative	Positive	
Age			0.666
< 60	14 (48.28%)	15 (51.72%)	
≥ 60	26 (43.33%)	34 (56.67%)	
Gender			0.182
Female	16 (55.17%)	13 (44.83%)	
Male	24 (40.00%)	36 (60.00%)	
Tumor diameter			0.040
< 5 cm	12 (66.67%)	6 (33.33%)	
≥ 5 cm	28 (39.44%)	43 (60.56%)	
Tumor localization			0.081
Upper 1/3	10 (58.82%)	7 (41.18%)	
Middle1/3	9 (27.27%)	24 (72.73%)	
Lower 1/3	9 (56.25%)	7 (43.75%)	
Whole stomach	12 (52.17%)	11 (47.83%)	
Histological type			0.023
Adenocarcinoma	19 (35.19%)	35 (64.81%)	
Adenocarcinoma mucinosum	21 (60.00%)	14 (40.00%)	
Histological differentiation			0.002 (< 0.001)
Moderately differentiated	5 (20.00%)	20 (80.00%)	
Poorly differentiated	15 (42.86%)	20 (57.14%)	
Non differentiated	20 (68.97%)	9 (31.03%)	
Depth of invasion			0.208
T1	5 (83.33%)	1 (16.67%)	
T2	2 (28.57%)	5 (71.43%)	
T3	28 (42.42%)	38 (57.58%)	
T4	5 (50.00%)	5 (50.00%)	
Lymph node metastasis			0.071
Absent	11 (64.71%)	6 (35.29%)	
Present	29 (40.28%)	43 (59.72%)	
Distant metastasis			0.794
Absent	28 (45.90%)	33 (54.10%)	
Present	12 (42.86%)	16 (57.14%)	
Blood vessel infiltration			0.980
Absent	21 (43.75%)	27 (56.25%)	
Present	4 (44.44%)	5 (55.56%)	
Lymphatic vessel infiltration			0.358
Absent	10 (47.62%)	11 (52.38%)	
Present	16 (35.56%)	29 (64.44%)	
Perineural infiltration			0.628
Absent	13 (46.43%)	15 (53.57%)	

Present	22 (40.74%)	32 (59.26%)	
Peritumoral inflammation			0.453
Absent	19 (46.34%)	22 (53.66%)	
Present	16 (38.10%)	26 (61.90%)	
Desmoplasia			0.710
Small	24 (43.64%)	31 (56.36%)	
Large	11 (39.29%)	17 (60.71%)	
Helicobacter pylori infection			0.824
Absent	26 (42.62%)	35 (57.38%)	
Present	10 (45.45%)	12 (54.55%)	
Lauren's classification			0.020
Intestinal	13 (29.55%)	31 (70.45%)	
Diffuse	20 (55.56%)	16 (44.44%)	

[a]In bracket is *P* value before Dunn's multiple comparison post hoc test.

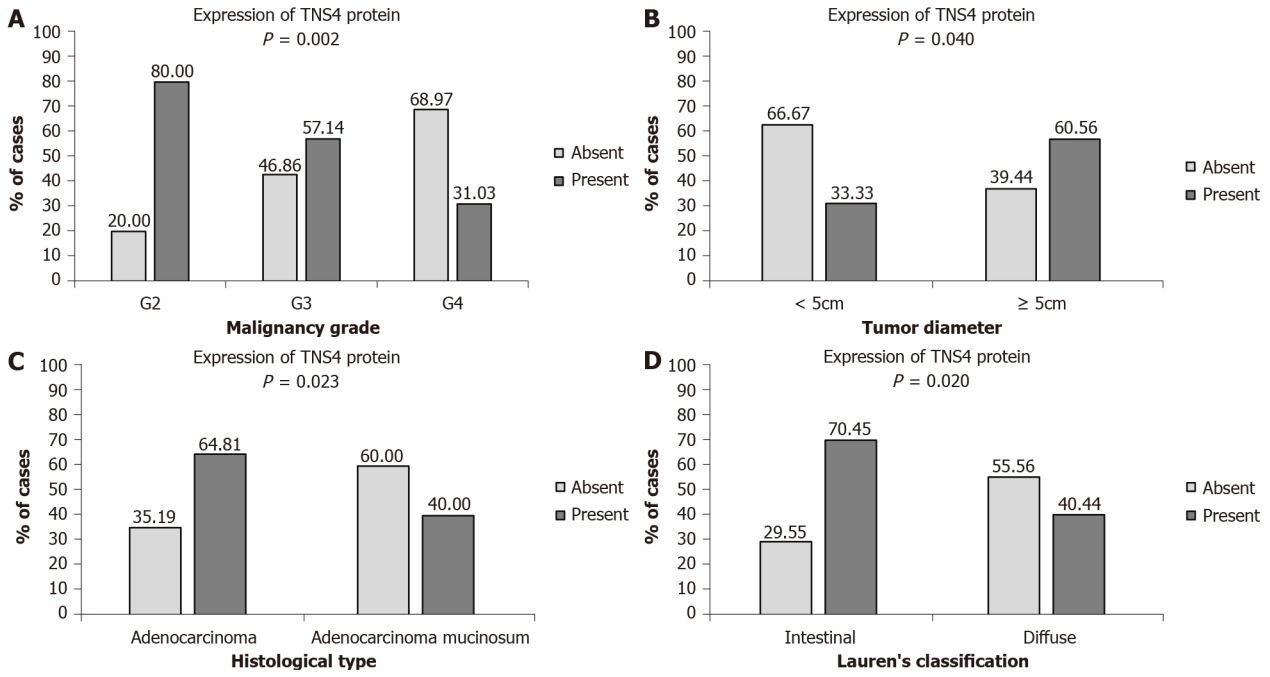

Figure 2 Tensin 4 expression with clinical and pathological parameters of gastric cancer. A: Comparison of tensin 4 (TNS4) expression and (A) malignancy grade, G2 *vs* G4, statistically significant at $P = 0.002$; B: Tumor diameter; C: Histological type; D: Lauren's classification. The comparison of TNS4 expression with the selected clinicopathological parameters was conducted using the Mann-Whitney U test for two groups and the Kruskal-Wallis test for three groups. Dunn's multiple comparison post hoc tests were implemented for the Kruskal-Wallis test. A value of $P < 0.05$ was considered significant. [a]$P < 0.05$ G2 *vs* G4 TNS4 expression level in gastric cancer. G2: Moderately differentiated gastric cancer (GC); G3: Poorly differentiated GC; G4: non-differentiated GC.

carcinoma tumors[11,20]. This finding indicates a correlation between TNS4 expression and the progression of the tumor and may be associated with the activation of signaling pathways, including PI3/AKT and Ras/MAPK, the stimulation of which intensifies the processes of cell proliferation and migration[13]. During the activation of these pathways, TNS4 expression is increased under the influence of epidermal growth factor at the levels of mRNA transcription and protein translation. Increased TNS4 expression leads to enhanced migration and proliferative potential of cancer cells.

We observed significant correlations between the expression of TNS4 and malignancy grade, histological type and type of tumor according to Lauren's classification. TNS4 protein was much more frequently present in moderately differentiated

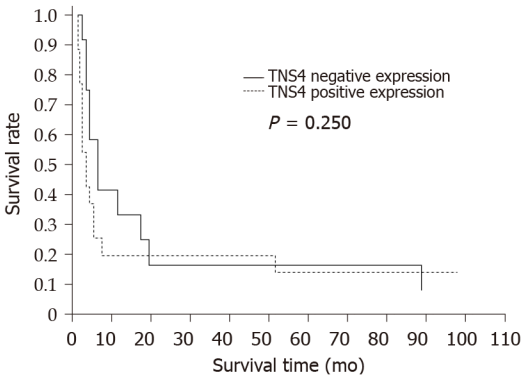

Figure 3 Analysis of the correlation of tensin 4 protein expression with the overall survival rate.

tumors than in poorly differentiated and non-differentiated tumors, in adenocarcinomas without mucinous components and in the intestinal type of GC. The classification of GC based on the degree of glandular cell differentiation is a prognostic factor. The more differentiated the gastric adenocarcinoma, the better the prognosis is for a patient. Poorly and non-differentiated adenocarcinoma cells differ phenotypically from each other, forming clusters without visibly formed glands[21]. There are no reports in the literature suggesting a significant relationship between TNS4 protein expression and the grade of tumor malignancy, which indicates the uniqueness of our study. In contrast, Sakashita *et al*[19] evaluated the *TNS4* mRNA expression and observed that patients with moderately or poorly differentiated GC had higher levels of *TNS4* expression. The differences in the results obtained by these researchers and us may be due to numerous mutations in the *TNS4* gene in tumor cells, entailing changes in the structure of this protein. We demonstrated higher expression of this protein in moderately differentiated gastric adenocarcinoma, which suggests that TNS4 overexpression is associated with a better prognosis of the tumor.

Poorly differentiated GCs are characterized by weak or no developed glandular tubules. Under normal conditions, TNS4 is involved in glandular duct morphogenesis. This phenomenon was described by Wu *et al*[22] after their study on a 3D model of RWPE-1 cells of the prostate. These researchers demonstrated that a decrease in protein expression does not affect the phenotype of the newly formed glands, although their number decreased. The authors suggested that lowering TNS4 expression inhibits the proliferation of normal epithelial cells of the prostate by stopping the cell cycle. However, increased expression of TNS4 leads to disturbances in the formation of glandular ducts through β_1-integrin/FAK pathway induction. Albasri *et al*[8] identified a link between TNS4 and the morphology of colorectal cancer cells. By means of confocal microscopy, these researchers demonstrated that induced expression of TNS4 affects the shape of cancer cells. Under the conditions of TNS4 overexpression, HCT116 cells assume a spindle shape compared to cells with physiologically low expression of TNS4.

In our research, we demonstrated significant differences in TNS4 expression between the two histological types of GC. We noted that positive expression of TNS4 protein was more frequent in GCs without mucinous components than in mucinous adenocarcinomas. Gastric mucosal adenocarcinomas have been linked to poorer prognosis, lower sensitivity to oncological treatment and increased risk of acquiring resistance to chemotherapy[23]. Currently, there are no data available to indicate a link between TNS4 expression and gastric adenocarcinomas without a mucinous component. Phenotypically, GC with a mucinous component is characterized by a considerable amount of mucin in the extracellular space (> 50%)[24].

In our study, we correlated the expression of TNS4 protein with the cancer type according to Lauren's classification. Statistical analysis showed that positive TNS4 staining correlates with the intestinal type of tumor. The presence of this protein was more often observed in adenocarcinomas of the intestinal than diffuse type. The results presented by Chen *et al*[25] indicate that the intestinal type correlates with better prognosis and better clinical and pathological parameters than the diffuse type. From a histological point of view, intestinal-type GC is phenotypically characterized by cells forming glandular ducts in the lumen of which the cancer grows. Usually, these cells do not secrete mucus, but if it is produced, it remains in the lumen of the glands[26]. In the literature, we did not find any studies assessing TNS4 expression in relation to the histological type of GC according to Lauren's classification. The positive

correlation between the expression of TNS4 and the intestinal type of cancer suggests further directions of research as well as the need to investigate the role of this protein in the histogenesis of GC.

Our study did not show any correlation between the level of TNS4 protein expression and tumor progression (pT, pN, pM status), although the literature shows that TNS4 is located in focal adhesion sites and participates in cell migration, thus increasing the metastatic potential of tumor cells. Qi *et al*[17] observed a significant correlation between TNS4 expression and the tumor stage. Their results indicated a correlation between TNS4 expression and deeper infiltrating tumors as well as lymph node and distant metastases. We did not observe similar significant relationships in our study. Other researchers have shown that stimulated TNS4 expression results in an increased ability of cells to migrate, infiltrate into adjacent tissues and metastasize. TNS4 was shown to play an important role in epithelial-mesenchymal transition by stimulating several signaling pathways, including E-cadherin, Akt, Src, TGF-β/Smad and Snail[8,17,27-29]. Other signaling pathways that are stimulated under TNS4 overexpression are PI3K/Akt and Ras/MAPK. Their activation may intensify cell proliferation and migration[13]. Furthermore, *in vitro* studies revealed that TNS4 may promote colony formation, as demonstrated in a study on pancreatic cancer cells[30]. Another important signaling protein in cancer development is MET, which is stabilized by TNS4 and acts as a mediator in carcinogenesis[31]. The exact mechanisms regulating TNS4, *e.g.*, a mutation that stabilizes this protein or amplifies the *TNS4* gene, have not been discovered thus far. Thorpe *et al*[32] reported that TNS4 can be regulated by the EGFR/KRAS and STAT3 pathways. Other studies have indicated that the expression of this protein may be modulated by growth factors and cytokines, *e.g.*, FGF2, PDGF, IGF-1, TGF-β, IL-6 and IL-13[33]. Other literature reports suggest that TNS4 may be engaged in tubulogenesis[34] as one of the stages of angiogenesis. The formation of blood vessel networks by tumor cells is the way in which the tumor microenvironment acquires nutrients and oxygen needed for tumor growth. Various publications have presented evidence for the oncogenic role of TNS4. In our research, we did not confirm the procancer role of the TNS4 protein. Our results prove that TNS4 may be related to the formation of a certain type of cancer, as observed in prostate cancer[35].

Moreover, we did not demonstrate any significant correlations between TNS4 expression level and the patients' overall survival rate. According to the works of Qi *et al*[17] and Sakashita *et al*[19], TNS4 overexpression correlates with shorter survival time and worse prognosis for patients with GC. In our study, the lack of this correlation may result from a small number of evaluated patients and the lack of data on the survival of many of these patients, which is a limitation of our research and does not allow us to draw broader conclusions about the participation of TNS4 in GC carcinogenesis. Additionally, it is possible that differences between the mRNA level in the study by Sakashita *et al*[19] and the protein expression level in our research are because the regulation of TNS4 transcription depends on the type of cells or tissues as well as on potential mutations in the TNS4 gene in GC cells. Thus, the expression level of this protein in different tissues is different. The control of this gene expression may also result from epigenetic modifications[36].

CONCLUSION

The expression of TNS4 was significantly higher in tumors with a diameter of ≥ 5 cm, of a moderately differentiated type of GC without a mucinous component, and of the intestinal type according to Lauren's classification. Increased levels of TNS4 expression are linked to the histological type of GC with a better prognosis.

ARTICLE HIGHLIGHTS

Research background

Gastric cancer (GC) is still one of the most common malignant neoplasms worldwide in terms of incidence and cancer motility. The development of GC is a multistage process. Tensin 4 (TNS4) belongs to the tensin family. This protein can participate in the transmission of intracellular signals. TNS4 plays an important role in biological processes connected with carcinogenesis, such as proliferation, migration, cell adhesion and invasiveness. It is very important to search for new biomarkers that

could help to diagnose GC at the early stages of its development.

Research motivation

We wanted to evaluate the role of the TNS4 protein in the development of GC. This protein may be a promising biomarker in the diagnosis of GC at the early stages of its development.

Research objectives

The study objective was to show the expression of TNS4 in GC tissues and assess the relationship between protein expression and clinical and pathological parameters and the overall survival rate of patients.

Research methods

In our study, we used immunohistochemistry to evaluate the expression of the TNS4 protein. The research was conducted on a group of 89 patients.

Research results

We observed that higher TNS4 expression was more often observed in GCs with a larger diameter (P = 0.040). Our results also showed that an increase in TNS4 expression was more frequently observed in tumors without mucinous components than in tumors from mucosal cancers (P = 0.023). Furthermore, higher TNS4 expression was demonstrated in moderately differentiated tumors (P = 0.002). Increased TNS4 expression was also noted in the intestinal type of GC according to Lauren's classification (P = 0.020). No significant correlation was found between the expression of TNS4 and the overall survival rate of patients.

Research conclusions

The expression of TNS4 was significantly higher in tumors with a diameter of ≥ 5 cm, of a moderately differentiated type of GC without a mucinous component, and of the intestinal type according to Lauren's classification. Increased levels of TNS4 expression are linked to the histological type of GC with a better prognosis.

Research perspectives

It is possible to develop this study with cell line methods. It will be possible to investigate the potential role of TNS4 in GC cell proliferation.

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ORIGINAL ARTICLE

Basic Study

Tumor irradiation may facilitate the detection of tumor-specific mutations in plasma

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Abstract

BACKGROUND

The mutation-based analysis of circulating tumor DNA (ctDNA) is a promising diagnostic tool for clinical oncology. However, it has low success rate because many cancer patients do not have detectable ctDNA in the bloodstream.

AIM

To evaluate whether preoperative tumor irradiation results in a transient increase of plasma ctDNA concentration due to the induction of apoptosis in radiation-

Whitehead AJ and Imyanitov EN prepared the manuscript; all authors approved the final version of the article.

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Conflict-of-interest statement: All authors have nothing to disclose.

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exposed cells.

METHODS
This study focused on patients with locally advanced rectal cancer, because preoperative tumor irradiation is a part of their standard treatment plan. Nine subjects, whose tumors contained *KRAS, NRAS* or *BRAF* mutations, donated serial blood samples 1 h prior to the first fraction of irradiation (at baseline), immediately after the first fraction (time 0), and 1, 3, 6, 12, 24, 36, 48, 72 and 96 h after the first fraction. The amount of mutated gene copies was measured by droplet digital PCR.

RESULTS
Five out of nine patients were mutation-negative by ctDNA test at baseline; two of these subjects demonstrated an emergence of the mutated DNA copies in the bloodstream within the follow-up period. There were 4 patients, who had detectable ctDNA in the plasma at the start of the experiment; three of them showed an evident treatment-induced increase of the content of mutated *RAS/RAF* alleles.

CONCLUSION
Local tumor irradiation may facilitate the detection of tumor-specific DNA in the bloodstream. These data justify further assessment of the clinical feasibility of irradiation-assisted liquid biopsy.

Key Words: Liquid biopsy; Rectal cancer; *KRAS*; *BRAF*; Mutations; Tumor response; Radiotherapy

Core Tip: The detection of circulating tumor DNA (ctDNA) in cancer patients is compromised by the low sensitivity of this assay. We hypothesized that tumor irradiation may lead to the transient increase of ctDNA content due to induction of cell death. Nine patients with locally advanced *RAS*/*RAF*-mutated rectal cancer provided serial blood samples at baseline and during the first 96 h after the first dose of tumor irradiation. Treatment-induced elevation of the concentration of mutated *RAS*/*RAF* alleles in the blood was revealed in five of these subjects. In conclusion, local tumor irradiation may facilitate the detection of plasma ctDNA and thus improve the efficacy of liquid biopsy.

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INTRODUCTION

"Liquid biopsy" is a popular diagnostic tool, which is based on the identification of tumor-specific markers in plasma or other body fluids. The analysis of several proteins, *e.g.*, prostate-specific antigen, carcinoembryonic antigen, CA-125 *etc.* has been utilized for years, however, these assays have significant limitations with regard to specificity and sensitivity[1,2]. The examination of tumor-derived mutations in circulating DNA (ctDNA) is considered to be more promising[3]. Indeed, some methods of genetic testing, for instance, droplet digital PCR (ddPCR) or next-generation sequencing allow the detection of a single mutated allele within a huge excess of wild-type nucleic acids[4,5]. In addition, while the protein-based liquid biopsy is not truly cancer-specific but rather tissue-specific, oncogenic mutations are strongly associated with the malignant phenotype. For the time being, clinical use of

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ctDNA tests is largely limited to the analysis of secondary mutations emerging during targeted therapy, as these assays may help to identify mechanisms of acquired drug resistance and therefore guide the subsequent treatment choice. It is anticipated that in the near future liquid biopsy will support other components of cancer care, such as screening, early diagnosis, analysis of treatment outcome and monitoring of relapse of tumor disease[6-11].

ctDNA-based liquid biopsy may have unacceptably low sensitivity. It appears that many categories of neoplasms (medulloblastomas; gliomas; cancers of kidney, thyroid, breast, *etc.*) are composed of relatively well-preserved cells, which do not shed DNA in the bloodstream, at least when the tumor is small[12,13]. Consequently, the analysis of ctDNA cannot substitute the detection of mutations in tumors tissue, *i.e.*, tissue biopsy cannot be easily replaced by liquid biopsy. The development of tools, which allow non-invasive examination of tumor characteristics, is of great value. One of the options may involve utilization of various agents, which induce tumor cell death and thus facilitate DNA shedding. In particular, tumor irradiation may increase tumor-specific ctDNA level due to the involvement of the above mechanism[14-16].

While considering the appropriate model for the validation of this assumption, we found it reasonable to focus on rectal cancer. Approximately 40%-50% of rectal carcinomas contain missense mutations in *KRAS, NRAS* or *BRAF* oncogenes, which can be used for ctDNA assays[17-19]. Furthermore, many rectal cancer patients undergo preoperative radiotherapy (RT) as a part of the treatment plan. Here we present the results of the study, which involved consecutive patients with mutation-positive rectal cancer. We demonstrate, that tumor irradiation indeed results in a transient increase of concentration of tumor-derived DNA and thus can be considered as a liquid biopsy supporting tool.

MATERIALS AND METHODS

Study workflow

The study considered treatment-naive patients with histologically verified locally advanced rectal cancer (T1-2/N1-2/M0, T3-4/N0-2/M0), who were referred to the St.-Petersburg City Cancer Center between February 2019 and April 2020 and who planned to undergo preoperative RT. The study was approved by the local Ethics Committee. Thirty patients provided informed consent and underwent *RAS/RAF* mutation testing (Figure 1). Thirteen analyzed tumors carried nucleotide substitutions in the mentioned genes. Four subjects failed to participate in the study due to various reasons (two tumors contained "rare" *RAS* mutations (*KRAS A59G* and *NRAS G12C*), which could not be detected by available ddPCR assays; 1 patient experienced rapid disease progression and was not subjected to RT; 1 patient developed acute parotitis and went to another hospital for treatment). Finally, nine patients were included in the ctDNA study and provided serial blood samples. Clinical characteristics of invited patients are summarized in the Supplementary Table 1.

RT and response evaluation

RT was performed according to routine procedures either with 45–50 Gy in 25–28 fractions or short-course radiation therapy (25 Gy in 5 fractions) with or without concurrent fluoropyrimidine-based chemotherapy. Chemotherapy was delivered according to standard regimens (capecitabine 825 mg/m^2 twice daily given within 5 d per week for 6-8 weeks). After RT patients were restaged with magnetic resonance imaging and the response was evaluated according to the TRG (tumor regression grade) system[20]. When the patients were surgically treated, the pathologic response was evaluated according to Mandard criteria[21]. The treatment results summary is presented in Supplementary Table 2.

Sample collection and processing

Patients provided blood at 11 different time points: 1 h before the first fraction of radiation (at baseline), immediately after the first fraction (time 0), and 1, 3, 6, 12, 24, 36, 48, 72 and 96 h after the first fraction (Figure 2). Ten milliliters of blood were collected into PAXgene Blood ccfDNA Tubes (Qiagen) or cf-DNA/cf-RNA Preservative Tubes (Norgen). Plasma samples were separated from the cellular fraction within 2-8 h after the blood-draw by two-step centrifugation (400 g for 10 min at room temperature followed by 14400 *g* for 10 min at 4 °C). The supernatants were aliquoted into 2 mL tubes and stored at −70 °C until further use. Cell-free DNA was extracted with the QIAmp Circulating Nucleic Acid Kit (Qiagen) as recommended by

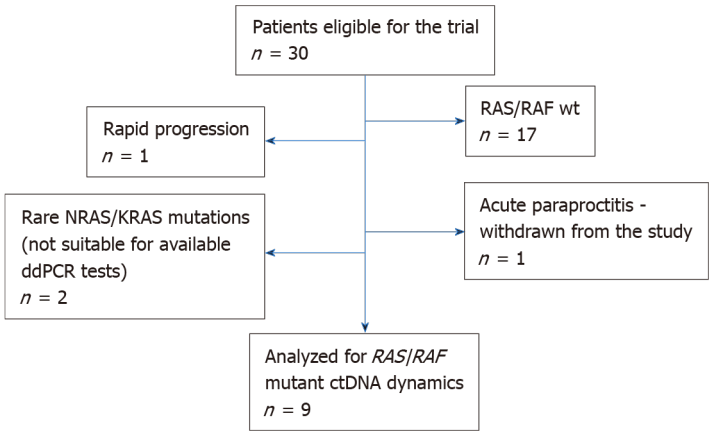

Figure 1 The flowchart of patients screening.

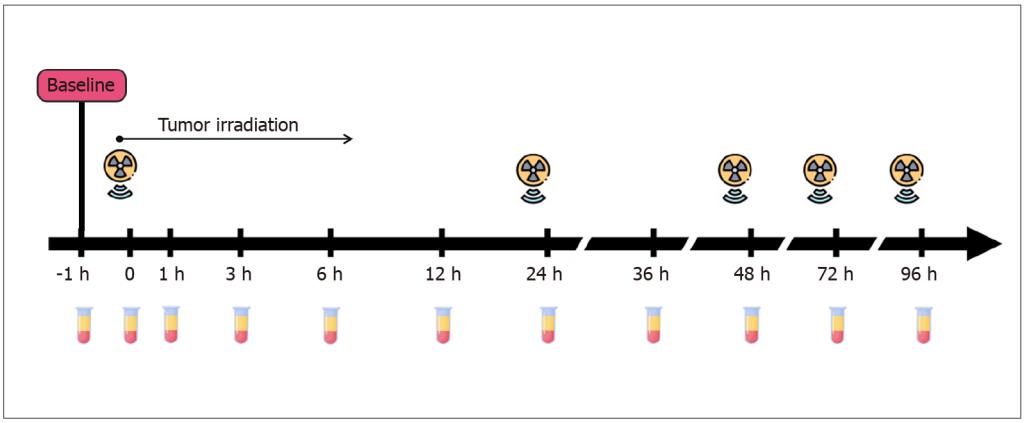

Figure 2 Schedule for serial blood-takes and irradiation fractions.

Diefenbach *et al*[22]. Isolated DNA was subsequently diluted in sterile distilled water and frozen at −24 °C until further analysis.

ctDNA analysis

The fractions of *KRAS/NRAS* mutations in codons 12, 13, 61 or *BRAF V600E* allele were measured by ddPCR using the QX100 Bio-Rad System[23]. ddPCR reactions were performed in triplicate. These reactions contained 2X ddPCR Supermix for Probes (no UTP, Bio-Rad), mutation-specific oligonucleotides (see Supplementary Tables 3 and 4) and 2-3 μL of the template DNA in a total reaction volume of 22-23 μL. Data analyses were performed with the QuantaSoft Software version 1.7.4 as recommended by the manufacturer. All ddPCR reactions, which yielded 10 or more droplets with the target DNA molecule, were considered informative. The absolute number of tumor-derived "mutated" DNA copies in 1 mL of plasma (C_{mut}) was calculated according to the formula:

$$\text{N mut copies/1 mL plasma} = \frac{\text{Concentration}\left(\frac{\text{copies}}{\mu\text{L}}\text{cfDNA}\right) \times \text{V templat}}{\text{V plasma}}$$

Where: Concentration – number of «mutated» droplets per 1 μL of ddPCR reaction; $V_{template}$ – volume of ctDNA aliquot taken into ddPCR, μL; $V_{dilution}$ – total volume of diluted ctDNA sample collected from the plasma, μL; V_{plasma} – volume of processed plasma, mL.

Statistics

The change of ctDNA content after tumor irradiation was evaluated according to the following formula:

$$\text{Percentage change (\%)} = 100\% \times \frac{\text{final Cmut} - \text{initial Cmut}}{\text{initial Cmut}}$$

Quantitative data were present as a median values/range or means ± 95% confidence interval (1.960σx). The non-parametric Wilcoxon Signed Rank Test and Mann–Whitney *U* test were utilized to compare the medians. *P* value of < 0.05 was considered statistically significant. All calculations were performed using IBM SPSS v.23 software package.

RESULTS

Nine rectal patients were included in the study of ctDNA fluctuations occurring within the first hours after RT. Individual characteristics of the patients are given in Table 1. Four out of nine (44%) analyzed subjects had detectable *RAS*/*RAF* mutations in plasma DNA at baseline (at least 5 mutation-specific signals per reaction). The probability of detecting ctDNA in plasma did not correlate with any clinical characteristics, *e.g.* age, gender, mutation type, T/N stages, tumor grade, tumor location within the rectum, extramural venous invasion, circumferential resection margin, tumor response to treatment or PFS (statistical data not shown).

Three of 5 patients, who were negative for plasma *RAS*/*RAF*-mutated DNA at baseline, did not show the presence of mutated copies in subsequent serial samples obtained after tumor irradiation. The remaining two patients (#ArAS and #GaZM) demonstrated an appearance of the mutated DNA copies within the follow-up period (Table 2 and Figure 3).

Four patients were recognized as "plasma-positive" at baseline. The mean concentration of mutated copies in plasma samples was 82 copies per 1 mL (22, 23, 39, and 244, respectively). The variant allele frequency (VAF) of circulating mutations ranged from 0.5% to 27.2%. The analysis of changes in mutated ctDNA concentration occurring within the first 96 h of treatment revealed a pronounced increase in the number of circulating *RAS*/*RAF* mutant copies in patients #DaKS, #ArTP, #MaNK (with the maximum percent changes equal to 509%, 174% and 71%, respectively). Patient #MaLI showed less consistent variations in ctDNA content, with a maximal concentration at the start (23 mutant copies per 1 mL plasma, VAF 2.3%) and a number of subsequent spikes and drops (Figure 3).

There was no correlation between the content of mutated ctDNA and the total irradiation dose accumulated during the blood collection time (r = -0.400; P (2-tailed) = 0.253, Spearman's Rho).

DISCUSSION

Patients with locally advanced rectal cancer provide a good opportunity for the analysis of RT-induced changes in the ctDNA level, as these malignancies frequently contain *RAS*/*RAF* mutations and the tumor irradiation is a part of routine clinical management of this disease[21]. The data obtained within this study are consistent with prior investigations, which were performed on lung cancer patients and demonstrated that radiotherapeutic or chemoradiotherapeutic intervention may result in a transient increase of the level of ctDNA in some cases[14-16]. As compared to published reports[14-16], our study considered multiple evenly distributed time points within the first day after tumor irradiation. We anticipated, that this additional effort may help us to identify a time interval characterized by maximal RT-induced ctDNA release. However, there was a significant interpatient variability with regard to the

Table 1 Patients, tumors and treatment characteristics

Patient ID	Gender	Age	cT	cN	Stage	CRM	EMV	Tumor location	RAS/RAFstatus	Total RT dose, Gy	Chemosensibilization[1]	RECIST	Surgery	MRI TRG	Mandard TRG	ypT	yN	Progression[2]	Follow-up, mo	ctDNA positive at baseline
																		Yes/no		
ArAS	M	44	3	1	3	No	No	L	KRAS G12S	50	Yes	SD	Yes	III	NA	3	0	No	6.90	No
GaZM	F	66	3	2	3	Yes	No	U-M	KRAS G13D	46	Yes	PR	Yes	IV	4	4	1	No	4.03	No
DaKS	M	73	4	1	3	Yes	No	L	KRAS G12A	25 (short course)	No	PD	No	IV	NA	NA	NA	Yes	4.73	Yes
ArTP	F	81	4	1	3	Yes	Yes	L	KRAS Q61L	50	No	SD	Yes	III	3	3	0	Nd	5.80	Yes
MaLI	F	78	3	1	3	No	No	L	NRAS G12D	25(short course)	No	PD	No	IV	NA	NA	NA	Yes	3.97	Yes
MaNK	F	48	3	2	3	No	Yes	M	KRAS G12D	50	Yes	SD	Yes	III	2	3	2	No	6.57	Yes
ZuNM	F	63	3	2	3	No	No	L-M	BRAF V600E	44	Yes	PR	Yes	II	2	3	0	No	5.13	No
MiMF	F	74	3	1	3	No	Yes	M	NRAS G12D	25(short course)	No	PR	No	III	NA	NA	NA	No	3.13	No
SaVV	M	65	3	1	3	Yes	No	L-M	NRAS Q61R	50	Yes	SD	No	III	NA	NA	NA	Nd	1.13	No

[1]Capecitabine 825 mg/m^2 twice daily was delivered on the days of RT (D1-5, D8-12, D 15-19, D22-26, D29-33).

[2]Disease status at March 1,.2021; Nd – lost from follow-up.

Tumor localization: U: Upper rectum, M: Middle rectum, L: Lower rectum; EMV: Extramural venous invasion; CRM: Circumferential resection margin; TRG: Tumor regression grade; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; NA: Not evaluated; ctDNA: Circulating tumor DNA.

timing of ctDNA concentration peaks (Table 2 and Figure 3).

There are several limitations of this investigation. Human studies involving multiple serial blood takes are logistically complicated and need to be well balanced with ethical issues, therefore it is understandable that our study and similar reports [14-16] are of limited size. Furthermore, the range of "natural" variations of ctDNA measurements occurring due to imperfect reproducibility of laboratory protocols or physiological fluctuations of ctDNA content is largely unknown. Therefore, although our study demonstrated a trend towards the RT-induced increase of ctDNA concentration in some rectal cancer patients, it is not clear how these observations need to be adjusted for the described above confounding factors. This limitation is also applicable to other published data sets[14-16].

The analysis of tumor-specific mutations at the initial diagnostic work-up is usually not complicated, given that the management of cancer patients always requires morphological visualization of transformed cells and thus implies the availability of malignant tissue. However, the detection of actionable mutations acquired during the

Table 2 Changes in circulating tumor DNA content during radiotherapy for locally advanced rectal cancer

Patient ID	Mutation	Baseline mutated ctDNA	ctDNA analysis	-1 h	0	1 h	3 h	6 h	12 h	24 h	36 h	48 h	72 h	96 h	Radiation dose per day / number of fractions / total dose accumulated for the blood collection time
ArAS	KRAS G12S	Neg	C (mut)[1]	0	0	0	3	0	0	0	0	14	12	nd	2.0 Gy/4 fr/8 Gy + capecitabine[3]
			C (mut + wt)[2]	642	1006	1337	1963	633	906	829	762	4298	3088	nd	
			VAF, %	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.4	nd	
GaZM	KRAS G13D	Neg	C (mut)	0	0	0	26	37	59	40	50	46	5	80	2.0 Gy/5 fr/10 Gy + capecitabine[3]
			C (mut + wt)	6	7	8	866	808	1450	604	441	506	112	1219	
			VAF, %	0.0	0.0	0.0	3.0	4.6	4.1	6.6	11.3	9.1	4.5	6.6	
DaKS	KRAS G12A	Pos	C(mut)	22	22	52	134	42	33	32	102	27	65	36	5.0 Gy/5fr/25 Gy
			C (mut + wt)	295	118	655	1802	588	292	170	549	359	391	331	
			VAF, %	7.5	18.6	7.9	7.4	7.1	11.3	18.8	18.6	7.5	16.6	10.9	
ArTP	KRAS Q61L	Pos	C (mut)	39	0	0	26	10	0	39	nd	42	107	nd	2.0 Gy/4 fr/8 Gy
			C (mut + wt)	7606	9430	4036	5028	3187	3033	12574	nd	10622	14443	nd	
			VAF, %	0.5	0.0	0.0	0.5	0.3	0.0	0.3	nd	0.4	0.7	nd	
MaLI	NRAS G12D	Pos	C (mut)	23	15	0	0	0	5	13	15	10	21	15	5.0 Gy/5 fr/25 Gy
			C (mut + wt)	981	1011	1073	1303	1309	1160	1010	724	815	673	1088	
			VAF, %	2.3	1.5	0.0	0.0	0.0	0.0	1.3	2.1	1.2	3.1	1.4	
MaNK	KRAS G12D	Pos	C (mut)	244	257	nd	nd	nd	Nd	254	335	415	418	387	2.0 Gy/5 fr/10 Gy + capecitabine[3]
			C (mut + wt)	897	1091	nd	nd	nd	Nd	1424	1832	2241	1719	1955	
			VAF, %	27.2	23.6	nd	nd	nd	Nd	17.8	18.0	18.5	24.3	19.8	
ZuNM	BRAF V600E	Neg	C (mut)	0	0	0	0	0	0	0	0	0	0	0	2.0 Gy/5 fr/10 Gy + capecitabine[3]
			C (mut + wt)	1652	372	522	522	522	522	671	837	1004	791	2805	
			VAF, %	0	0	0	0	0	0	0	0	0	0	0	
MiMF	NRAS G12D	Neg	C (mut)	0	0	0	0	0	0	0	0	0	0	0	5.0 Gy/5 fr/25 Gy
			C (mut + wt)	594	500	740	2294	804	681	681	386	644	2891	1536	
			VAF, %	0	0	0	0	0	0	0	0	0	0	0	
SaVV	NRAS Q61R	Neg	C (mut)	0	0	0	0	0	0	0	0	0	0	0	2.0 Gy/5 fr/10 Gy + capecitabine[3]

C (mut + wt)	196	237	541	840	621	748	353	372	272	261	249
VAF, %	0	0	0	0	0	0	0	0	0	0	0

[1]C (mut): Number of mutated copies per 1 mL of plasma measured by droplet digital PCR.
[2]C (mut + wt): Total number of target fragment (both *wt* and *mut*) copies per 1 mL of plasma measured by droplet digital PCR.
[3]Capecitabine 825 mg/m^2 twice daily was delivered on the 1-5 d of RT.
ctDNA: Circulating tumor DNA; VAF: Variant allele frequency.

course of therapy presents a challenge. For example, the management of lung cancer patients, whose tumors progressed during gefitinib, erlotinib or afatinib treatment, involves the analysis of EGFR T790M mutation. The presence of this mutation justifies the administration of osimertinib, while the absence of this substitution calls for other treatment options. Re-biopsy of multiple visceral tumor lumps is often not feasible; therefore, the analysis of EGFR T790M mutation usually relies on liquid biopsy[24,25]. Clinical studies demonstrate that the detection of EGFR T790M mutation in plasma is seriously compromised by the low sensitivity of the test, especially in patients with limited tumor burden[26,27].

This study utilized patients with localized rectal cancer, who had a moderate volume of tumor masses. It is therefore explainable that only 4 out of 9 patients had detectable ctDNA at baseline. These data are comparable with the results obtained in other studies[12,28]. We deliberately focused on rectal cancer disease, as these patients often receive irradiation during the standard preoperative treatment, so no additional interventions were involved within this investigation. We demonstrated that two out of five subjects, who were initially ctDNA-negative, showed the presence of mutated DNA copies in the plasma after the start of the therapy. In addition, 3 out of 4 initially ctDNA-positive subjects experienced a RT-related increase of ctDNA content. The obtained data look promising, so further studies, which involve tumor irradiation not as a part of regular treatment plan, but as an additional intervention aimed to support ctDNA analysis, appear to be justified.

The clinical utility of this approach deserves to be evaluated in lung cancer patients, who demonstrate the disease progression during the therapy by first- or second-generation EGFR inhibitors and therefore require the diagnostic detection of EGFR T790M substitution. It is feasible to organize a prospective study, where the tumor lumps observed in these patients will be subjected to irradiation in order to provoke the release of tumor DNA in the bloodstream. It is essential to minimize the risks of this procedure by considering the anatomic location of targeted tumor foci (particularly, the vicinity of large blood vessels), ensuring a highly precise topical delivery of the irradiation dose and accounting for potentially significant comorbidities. If this intervention was to increase the rate of EGFR T790M allele detection in the plasma while being sufficiently safe, the proposed approach would have significant potential for clinical use.

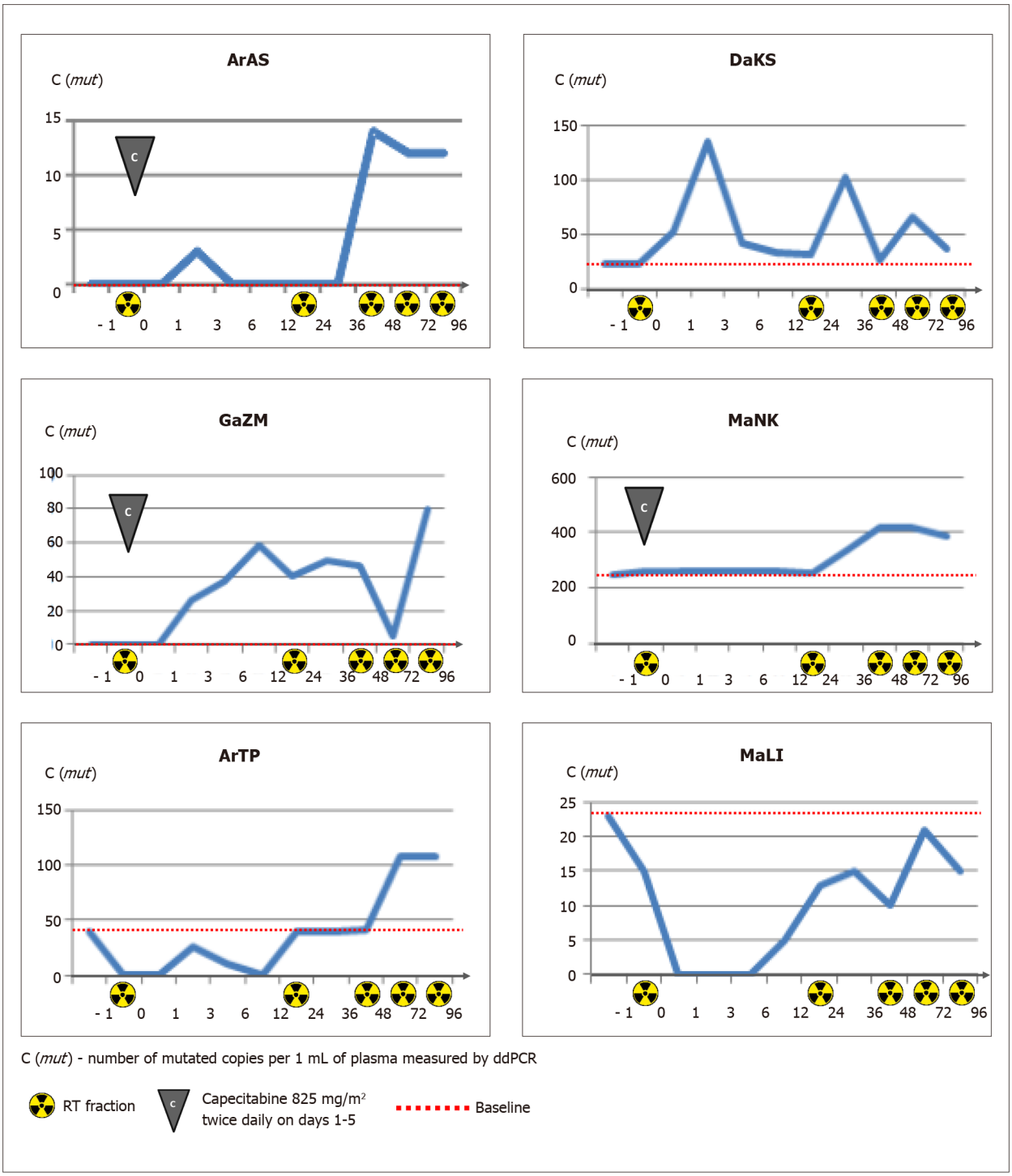

Figure 3 Changes in ctDNA content occurring within first 96 h after the start of radiotherapy.

CONCLUSION

Local tumor irradiation may facilitate the detection of plasma ctDNA. This study calls for a comprehensive evaluation of the clinical feasibility of irradiation-assisted liquid biopsy.

ARTICLE HIGHLIGHTS

Research background

The detection of circulating tumor DNA (ctDNA) is a valuable diagnostic tool,

however many cancer patients do not have detectable amount of ctDNA in their plasma.

Research motivation

We evaluated whether tumor irradiation may provoke the release of tumor DNA in the bloodstream and thus improve the efficiency of liquid biopsy.

Research objectives

We have chosen for the study patients with locally advanced rectal cancer as they usually receive preoperative tumor irradiation as a part of standard treatment plan.

Research methods

The study included 9 patients with *RAF/RAF* mutations. Multiple serial blood draws were taken within first 96 h after the first fraction of radiotherapy. The amount of mutated *RAF/RAF* copies in the plasma was quantified by the droplet digital PCR.

Research results

Five out of nine patients demonstrated increased ctDNA content at least at some plasma samples obtained after the beginning of radiotherapy.

Research conclusions

Radiotherapy is a promising tool for the improvement of performance of liquid biopsy.

Research perspectives

It is feasible to extend this study to lung cancer patients, who receive tyrosine kinase inhibitors and may experience acquired tumor resistance due to the gain of secondary mutation.

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SYSTEMATIC REVIEWS

Mixed odontogenic tumors: A review of the clinicopathological and molecular features and changes in the WHO classification

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Abstract

BACKGROUND

Ameloblastic fibromas and ameloblastic fibrosarcomas are rare odontogenic tumors, and controversy exists in the classification of cases presenting hard-tissue production: Ameloblastic fibrodentinoma (AFD) and ameloblastic fibro-odontoma (AFO). These cases are currently considered "developing odontomas" (hamartomatous lesions).

AIM

To analyze the clinicopathologic features of these lesions and discuss the changes in the 2017 World Health Organization classification.

METHODS

An electronic literature search was performed in the PubMed/MEDLINE database. An electronic search of the English language literature was performed and last updated in September 2020 in the PubMed/MEDLINE database using the following terms: "ameloblastic fibroma", "ameloblastic fibrodentinoma", "ameloblastic fibro-odontoma", "ameloblastic sarcoma", "ameloblastic fibrosarcoma", "ameloblastic fibrodentinosarcoma", "ameloblastic fibroodontosarcoma" and "odontogenic carcinosarcoma". The inclusion criteria were odontogenic tumor series, case reports and systematic reviews that provided sufficient clinical, radiological and microscopic documentation to confirm the diagnosis.

RESULTS

The database search strategy resulted in 947 papers. Articles focusing on other

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

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topics, articles that were not in English, duplicate articles, and articles without fulfilling the inclusion criteria were excluded. Finally, 96 publications were included in this review to describe and discuss the main features of the searched entities. Several aspects of AFO and AFD, such as biological behavior, age of occurrence, amount of hard tissue, and potential for malignant transformation into odontogenic sarcomas, support the neoplastic nature in most of the reported cases. Considering the clinical, radiographic, histopathological and molecular characteristics of odontogenic lesions with hard tissue production, we suggest that these types of lesions should continue to be recognized as odontogenic tumors by maintaining the classically used terms.

CONCLUSION
This recommendation will be relevant for future clinical, microscopic, and molecular studies to better understand the biology of these interesting odontogenic tumors.

Key Words: Ameloblastic fibroma; Ameloblastic fibrosarcoma; Odontogenic carcinosarcoma; Odontogenic tumors

Core Tip: We consider that the recent 2017 World Health Organization classification does not clarify the subject when considering ameloblastic fibrodentinoma (AFD) and ameloblastic fibro-odontoma (AFO) as "developing odontomas". According to the clinical, radiographical, histopathological and molecular features of the cases reviewed, we suggest that AFD and AFO should continue to be considered benign neoplasms. Thus, the nomenclature of these mixed benign odontogenic tumors would be congruent with the classification of ameloblastic/odontogenic sarcomas. Additionally, further studies are warranted to compare these interesting odontogenic tumors and finally better clarify and understand their similarities and differences.

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INTRODUCTION

Ameloblastic fibroma (AF) is a rare, benign odontogenic tumor formed by odontogenic ectomesenchyme that resembles the dental papilla, has embedded epithelial strands and nests, and is similar to dental lamina and enamel organs but without the presence of hard tissues. If the lesion has dentinoid material, it must be denominated as ameloblastic fibrodentinoma (AFD); when it produces enamel/enamel matrix, it is known as ameloblastic fibro-odontoma (AFO), independent of the amount of hard tissue present[1]. In 2005, WHO classified AF and AFD together, making no distinctions regarding epidemiological and clinical features. Microscopically, the only difference between AF and AFD is the presence of dentinoid in the latter. AFO affects younger patients and has shown a better prognosis than AF/AFD[1]. However, the WHO classification of 2017 states that AF rarely produces dental hard tissues and cases formerly considered AFD/AFO rather represent developing odontomas[2].

This group of mixed odontogenic tumors (AF and related-lesions) histologically resembles different tooth formation stages, particularly when dentin and enamel are produced, sharing similar morphologic features with the so-called "developing odontoma", which is considered a tumor-like malformation or hamartoma by WHO. Nevertheless, unlike odontomas, these mixed tumors present characteristics that support the concept of a true neoplasm, such as biological behavior, age of occurrence, and well-documented cases of malignant transformation into odontogenic sarcomas, namely, ameloblastic fibrosarcoma (AFS), ameloblastic fibrodentinosarcoma (AFDS)

and ameloblastic fibro-odontosarcoma (AFOS). Moreover, the recent publication of a few reports of odontogenic carcinosarcomas led to its inclusion as a specific tumor by WHO in 2017[2-4].

This review is based on the WHO classification of 2005, because most of the literature is based on this nomenclature, and it was performed to analyze the clinicopathologic features of these lesions to discuss the changes in the 2017 WHO classification.

MATERIALS AND METHODS

An electronic search of the English language literature was performed and last updated in September 2020 in the PubMed/MEDLINE database using the following terms: "ameloblastic fibroma", "ameloblastic fibrodentinoma", "ameloblastic fibro-odontoma", "ameloblastic sarcoma", "ameloblastic fibrosarcoma", "ameloblastic fibrodentinosarcoma", "ameloblastic fibroodontosarcoma" and "odontogenic carcinosarcoma".

Previous cases that did not use the current terminology for these tumors, recently identified as AF, AFD, AFO, AFS, AFDS or AFOS, were also found and evaluated for possible inclusion.

The inclusion criteria were odontogenic tumor series, case reports and systematic reviews including AF, AFD, AFO, AFS, AFDS or AFOS, which provided sufficient clinical, radiological and microscopic documentation to confirm the diagnosis. Reports without this information were excluded.

RESULTS

The database search strategy resulted in 947 papers. Articles focusing on other topics, articles that were not in English, duplicate articles, and articles without fulfilling the inclusion criteria were excluded. Finally, 96 publications were included in this review to describe and discuss the main features of the searched entities.

AF

Clinical characteristics: This uncommon benign mixed odontogenic tumor occurs preferentially in children and young adults, with a mean age of 14.9 years, ranging from 7 wk to 57 years. Only 20% of the cases are diagnosed in patients older than 20 years. Considering all odontogenic tumors, AF represents only 0.6% to 3.1% of these neoplasms. Most of the cases affect the mandible, with a slight predilection for male patients, with a male/female ratio of 1.4:1. The size of AF when diagnosed varies from 0.7 to 16 cm (mean of 4.05 cm)[5].

Most cases present as painless jaw swelling or are discovered during routine radiographical examination due to delayed tooth eruption, eventually causing cortical expansion and facial asymmetry. Approximately 80% of AF involves the posterior region of the mandible but has also been found on the posterior maxilla and rarely in the anterior region of the jaws[5,6].

Radiographic features: Radiographically, AF presents as a well-defined, unilocular (56%) or multilocular (44%) radiolucent lesion, with regular and well-defined margins, typically sclerotic (94%). Tumors measuring less than 5 cm usually tend to be unilocular. Approximately 80% of cases are associated with a single or several unerupted teeth, usually of permanent dentition. Root resorption and cortical perforation are uncommon and described in 8.1% and 5.2% of cases, respectively[5,6].

Histopathology: Microscopically, AF is a mixed tumor with variable amounts of epithelial and ectomesenchymal components in different areas of the same lesion. The ectomesenchyme resembles the embryonic dental papilla, comprising a myxoid cell-rich stroma involving odontogenic epithelial elements that may present different patterns: epithelial strands, comprising a double layer of cuboidal cells (Figure 1A); cords with tooth bud-like projections of cuboidal cells (Figure 1B); epithelial follicles comprising a layer of peripheral tall columnar ameloblast-like cells and a central area, displaying more loosely arranged stellate/spindle-shaped cells, similar to the stellate reticulum of the enamel organ (Figure 1C); clefts of mesenchymal tissue surrounding follicular epithelial proliferation can be present (Figure 1D); and smaller epithelial rosette-like islands that resemble remnants of dental lamina may be observed

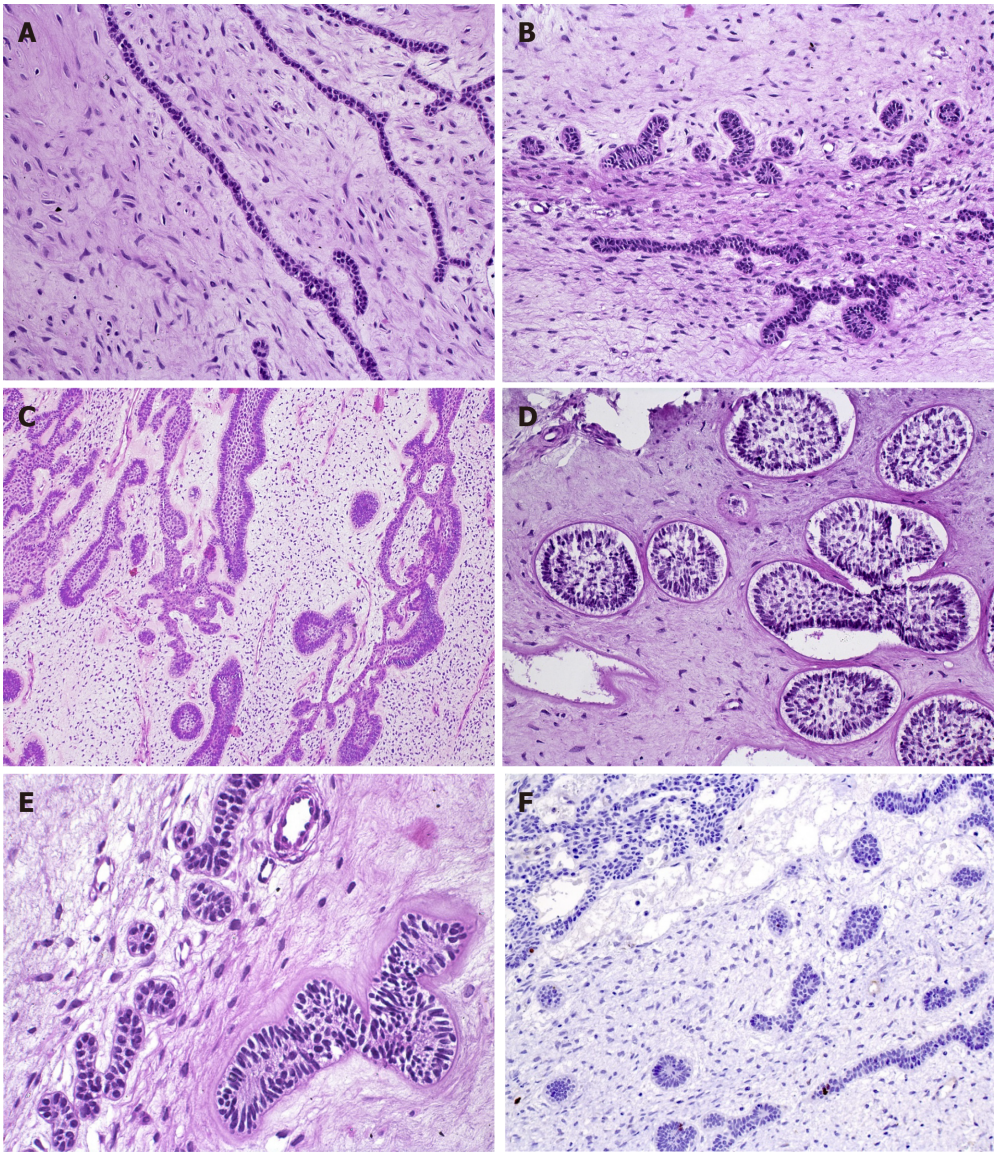

Figure 1 Diverse aspects of the odontogenic epithelium in ameloblastic fibromas within the cell-rich myxoid stroma. A: Epithelial strands, comprising a double layer of cuboidal cells (HE, 20×); B: Epithelial proliferation with primitive appearance that resembles tooth bud-like structures (HE, 20×); C: Epithelial component with a follicular pattern comprising columnar cells at the periphery of the nests with central stellate reticulum-like cells (HE, 10×); D: Clefts of mesenchymal tissue surrounding follicular epithelial proliferations (HE, 20×); E: Mild hyalinization surrounding the basal layer of the epithelial nest (left). Smaller epithelial rosette-like islands resemble remnants of dental lamina (right) (HE, 40×); F: A very low rate of proliferation in both mesenchymal and epithelial components, showing the benign behavior of ameloblastic fibromas (IHC for Ki-67, 20×).

(Figure 1E). Mitotic figures in either the epithelium or mesenchyme are uncommon, a finding consistent with the benign nature of the tumor. According to the 2005 WHO classification, no hard tissues, such as enamel or dentin, are present[1]. In the 2017 classification, AF rarely presented dental hard tissue formation that eventually reached an exceptional size[2]. According to the histopathological criteria of 2005, 280 cases of AF were identified in the literature. The proliferation rate is low, with a Ki-67 Labeling index generally lower than 3% (Figure 1F)[5-7].

Treatment and prognosis: Most reported AFs were treated conservatively by enucleation and curettage. Radical surgery is used in more extensive tumors or recurrent lesions. Recurrence was reported in 16.3% of cases, and malignant transformation into AFS was cited in 6.4%. Recurrence seems to be more common in younger patients and malignant transformation more common in older patients[5].

AFD

Clinical characteristics: AFD is a rare benign odontogenic tumor with histopathological features of AF and the formation of dysplastic dentin. The WHO classification

of 2005 describes AF and AFD together, without further considerations of the latter, beyond the presence of dentin/dentinoid. No strong evidence of differences in the biological behavior of AF and AFD is available[1,8]. However, the 2017 WHO classification of tumors cited that lesions referred to as AFDs are more likely "developing odontomas", and the editors suggest that they are no longer being considered mixed odontogenic tumors, as in the previous classification[2].

AFD is rare, corresponding to less than 1% of all odontogenic tumors in most reported series. It usually presents as asymptomatic swelling, more frequently at the posterior mandible (mandible/maxilla ratio of 2.4:1), often associated with a permanent unerupted tooth. When a deciduous tooth is involved, the lesions are generally located in the incisor area. From the 45 cases reviewed, we found a slight male predilection, corresponding to 59.5% of cases, usually in the first and second decades of life; however, 17 of 45 (37.7%) cases occurred in the third decade and beyond. The mean age was 17.8 years, with an age range of 1 to 63 years (Table 1).

Radiographic features: Radiographically, AFD presents as a well-defined radiolucency with varying degrees of radiopacity, depending on the amount of calcified dentinoid. In 2012, Giraddi and Garg[9] reported a large and aggressive AFD with irregular borders, with considerable expansion and perforation of the cortical bone; however, the possibility of eventual foci of malignant transformation to AFDS should be considered in this case.

Histopathology: Microscopically, AFD is formed by odontogenic epithelium and ectomesenchyme arranged in an indistinguishable pattern from AF, in addition to the presence of dentinoid (Figure 2A, B). The epithelial cords and islands resemble the dental lamina and enamel organ, lying in myxoid cell-rich ectomesenchymal tissue with stellate-shaped fibroblasts resembling dental papilla. The amount of dentinoid material is variable, but minimal evidence is sufficient for the diagnosis to be accepted [1]. We found only 45 cases of AFD in the English literature according to these characteristics. Similar to AF, the Ki-67 index in AFD is low in both epithelial and mesenchymal components[8].

Treatment and prognosis: The treatment of choice is surgical, with enucleation of the lesion and unerupted tooth involvement. Recurrence is uncommon (9%) and likely a consequence of incomplete surgical removal. Radical surgery has been used in aggressive, atypical or recurrent lesions[9]. AFD rarely progresses into ameloblastic fibrodentinosarcoma, in which only the mesenchymal component shows malignant transformation. Only 4 cases of AFDS with a preexisting benign lesion have been described in the English literature (Table 2).

AFO

Clinical characteristics: AFO is a slow-growing, expansive, benign mixed odontogenic tumor that is histologically similar to AFD but also contains enameloid material in variable amounts[1]. Similar to AFD, the term AFO was excluded from the latest WHO classification, in which lesions with these characteristics are considered developing odontomas[2].

According to the literature, AFO occurs mainly in children, with a mean age of 9.6 years. It has a male predilection, with a ratio of 1.85:1 and an average size of 3.3 cm, ranging from 0.8 to 14 cm. More than 80% of cases affect the posterior portion of the mandible, eventually causing facial asymmetry[10,11]. To our knowledge, 222 cases have been reported in the English literature, among which 211 were reviewed by Chrcanovic *et al*[5] and 11 additional cases were published later[12-22], including one peripheral case[23]. One case was associated with paresthesia of the chin and lower lip in a 12-year-old girl[22].

Radiographic features: AFO usually appears as a well-defined unilocular mixed radiolucent-radiopaque lesion, frequently in close association with the crown of an unerupted tooth. It commonly causes painless cortical expansion but no perforation [10,11].

We reviewed the literature and found 82 cases with optimal radiographic documentation, among which 22 (26.8%) presented radiographically as a single large opaque mass similar to odontoma and 11 (13.4%) presented several foci of opacities; however, most cases were poor in hard tissues, with 43 cases presenting few opacities (52.4%) and 6 cases appearing as radiolucent lesions (7.3%).

Histopathology: Similar to AF, AFO comprises odontogenic epithelium and ectomesenchyme, but it also contains hard dental tissues in variable amounts and degrees of

Table 1 Reported 45 cases of ameloblastic fibrodentinoma found in the English language literature

Case	Ref.	Year	Sex/age	Location
1	Straith[42]	1936	F/30	Posterior mandible
2	Field and Ackerman[43]	1942	NA/9	Posterior mandible
3	Stafne[44]	1943	F/25	Posterior mandible
4	Stafne[44]	1943	NA/23	Posterior mandible
5	Stafne[45]	1946	NA	NA
6	Thoma and Goldman[46]	1946	M/6	Maxillary sinus area
7	Ingham[47]	1952	F/19	Posterior mandible
8	Sirsat[48]	1952	M/36	Maxillary sinus area
9	Husted and Pindborg[49]	1953	M/4	Anterior maxilla
10	Husted and Pindborg[49]	1953	F/63	Posterior mandible
11	Hitchin and White[50]	1955	M/4	Anterior mandible
12	Pindborg[51]	1955	M/20	Posterior mandible
13	Gorlin *et al*[52]	1961	F/4	Anterior maxilla
14	Gorlin *et al*[52]	1961	F/13	Posterior mandible
15	Gorlin *et al*[52]	1961	M/8	Posterior mandible
16	Azaz *et al*[53]	1967	M/4.5	Anterior mandible
17	Manning and Browne[54]	1970	F/55	Posterior mandible
18	Hoggins and Browne[55]	1976	M/24	Posterior mandible
19	Gulmen *et al*[56]	1976	M/30	Anterior mandible
20	Godjesk *et al*[57]	1980	M/3.5	Anterior mandible
21	Rennie and Critchlow[58]	1981	M/7	Posterior maxilla
22	van Wyk and van der Vyver[59]	1983	M/8	Posterior mandible
23	Villafañe *et al*[60]	1986	F/22	Posterior maxilla
24	Lukinmaa *et al*[61]	1897	M/11	Posterior mandible
25	Anker and Radden[62]	1989	F/24	Posterior mandible
26	Ulmansky *et al*[63]	1994	M/60	Posterior maxilla
27	Ulmansky *et al*[63]	1994	M/8	Posterior maxilla
28	Cassidy *et al*[64]	1987	M/12	Posterior maxilla
29	Akal *et al*[65]	1997	M/9	Posterior mandible
30	Akal *et al*[65]	1997	M/22	Mandible
31	Takeda *et al*[66]	2000	M/21	Mandibular retromolar area
32	Karasu *et al*[67]	2004	F/21	NA
33	Bhargava *et al*[68]	2011	M/51	Anterior maxilla
34	Giraddi and Garg[9]	2012	F/17	Mandible
35	Bologna-Molina *et al*[8]	2013	F/1.5	Mandible
36	Sankireddy *et al*[69]	2013	M/14	Anterior maxilla
37	Salehinejad *et al*[70]	2013	F/13	Anterior mandible
38	Ikeda *et al*[71]	2014	F/8	Posterior mandible
39	Lee *et al*[72][1]	2014	F/4	Anterior mandible
40	Unsal *et al*[73]	2014	M/11	Anterior mandible
41	Joseph *et al*[74]	2015	M/12	Anterior Maxilla

42	Costa *et al*[75]	2015	F/12	Posterior mandible
43	Bhargava *et al*[76]	2016	M/1	Anterior mandible
44	Bavle *et al*[77]	2017	F/14	Posterior mandible
45	Sabu *et al*[78]	2018	M/20	Mandible (left body to right parasymphysis)

[1]Associated with calcifying cystic odontogenic tumor, only dentinoid production.
F: Female; M: Male; NA: Not available.

Table 2 Main data of 21 cases reported of ameloblastic fibrodentinosarcoma/ameloblastic fibro-odontosarcoma in the literature

Case	Ref.	Sex/age	Location	Mineralized tissues	Preexisting tumor	Progression
1	Villa[79]	F/20 yr	Posterior mandible	Enamel	Yes (NA)	Recurrence
2	Forman and Garret[80]	M/17 yr	Posterior mandible	Dentin and enamel	No	No recurrence
3	Altini and Smith[81]	M/27 yr	Mandible	Dentin	No	NA
4	Takeda *et al*[32]	M/19 yr	Maxilla	Dentin	AF	Recurrence and death
5	Howell and Burkes[31]	F/18 yr	Posterior mandible	Dentin and enamel	AFO	Recurrence, metastasis and death
6	Howell and Burkes[31]	M/36 yr	Posterior mandible	Dentin	AFO	Recurrence
7	Altini *et al*[41]	M/25 yr	Mandible	Dentin	No	No recurrence
8	Takeda *et al*[33]	M/23 yr	Mandible	Dentin and enamel	No	Recurrence and death
9	Corominas-Villafañe *et al*[82]	M/12 yr	Mandible	NA	AF	No recurrence
10	Herzog *et al*[83][1]	F/14 yr	Mandible	NA	AFO	NA
11	Bregni *et al*[25]	M/32 yr	Mandible	Dentin	No	NA
12	Muller *et a*[84]	M/83 yr	Mandible	Dentin and enamel	AFO	Recurrence
13	Zabolinejad *et al*[35]	M/4 mo	Maxillary sinus	Dentin	No	No recurrence
14	Mainenti *et al*[34]	F/12 yr	Mandible	Dentin and enamel	AFO	No recurrence
15	Wang *et al*[30]	F/45 yr	Posterior mandible	Dentin and enamel	No	No recurrence
16	Reiser *et al*[85]	F/6 yr	Mandible	Dentin and enamel	No	No recurrence
17	Khan *et al*[86]	F/17 yr	Mandible	NA	No	NA
18	Gatz *et al et al*[87]	F/14 yr	Maxilla	Dentin	AFO	Recurrence
19	Chen *et al*[88]	M/4 yr	Mandible	Dentin and enamel	No	No recurrence
20	Niu *et al*[89]	F/31 yr	Mandible	Dentin and enamel	No	No recurrence at 3 months, lost follow-up
21	Atarbashi-Moghadam *et al*[90]	F/32 yr	Mandible	Dentin	No	Recurrence and metastasis

[1]Article in German, abstract in English.
F: Female; M: Male; AF: Ameloblastic fibroma; AFO: Ameloblastic fibro-odontoma; AFDS: Ameloblastic fibrodentinosarcoma; AFOS: Ameloblastic fibroodontosarcoma; NA: Not available.

maturation, such as enameloid and dentinoid (Figure 2C-F). Frequently, Ki-67 is lower than 1% in epithelial and mesenchymal cells[1,24].

Treatment and prognosis: The treatment of choice is conservative surgery through enucleation and curettage, with removal of the unerupted tooth. Recurrence is uncommon, and malignant transformation is very rare, with only 6 cases reported to

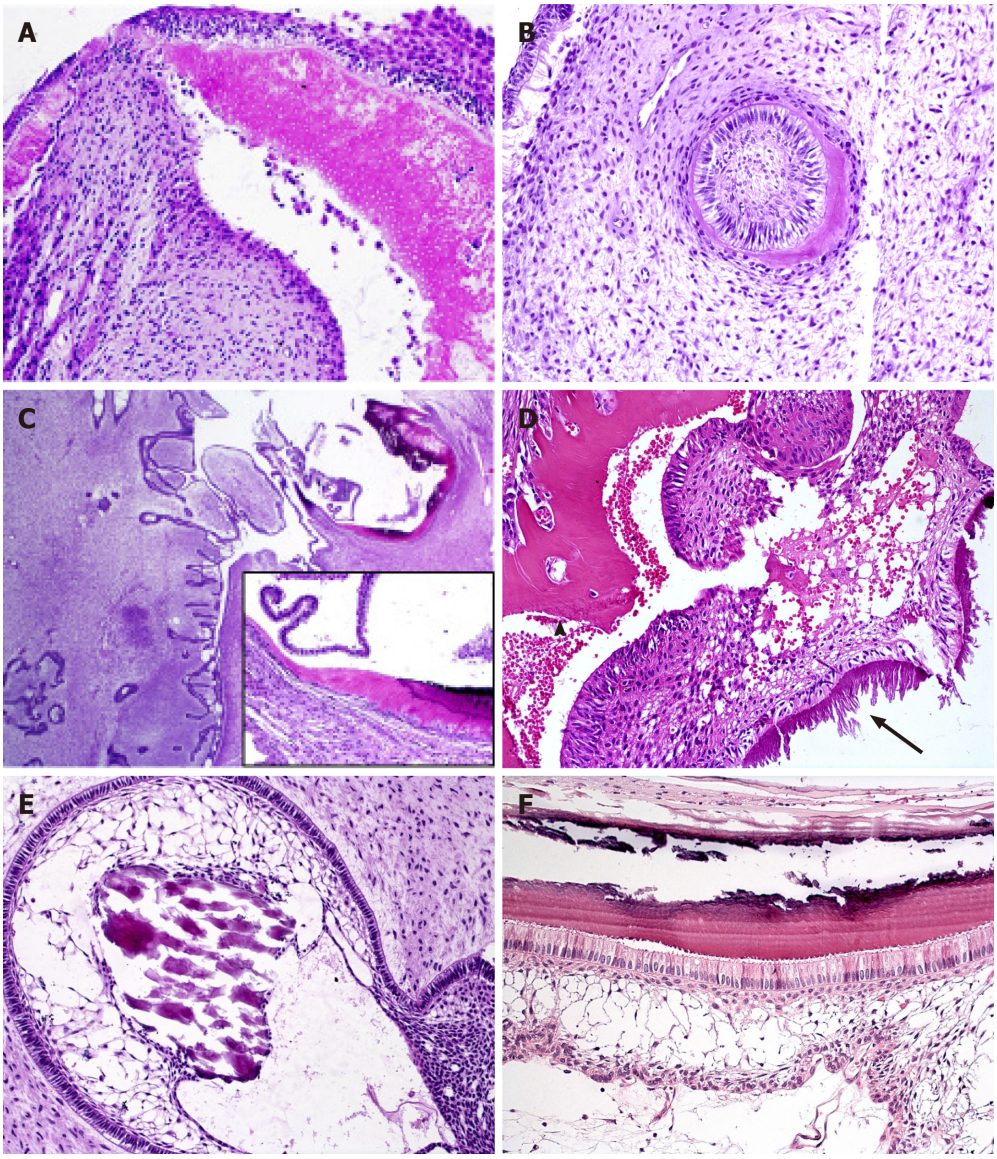

Figure 2 Mineralized tissue formation in ameloblastic fibrodentinoma and ameloblastic fibro-odontoma. A, B: Dentinoid induction by epithelial cells in ameloblastic fibrodentinoma; note the presence of tubules in (A) (HE, 20×); C: Prominent proliferation of soft tissue similar to ameloblastic fibromas and focal areas of dentinoid and enamel matrix production in close relationship with the epithelial component in ameloblastic fibro-odontoma (HE, 2.5×; inset 20×); D: Structures similar to tubules are observed in the dentinoid (arrowhead), which can be associated with odontogenic epithelium or ectomesenchymal tissue, while enamel matrix (arrow) associated with columnar odontogenic appears more basophilic, with different patterns of deposition that can resemble prisms or globules (HE, 20×); E: Calcificated material, compatible with enameloid, in direct relationship with epithelial cells of the stellate reticulum-like area (HE, 20×); F: Details of the columnar ameloblast-like cells with reverse polarization producing enamel matrix in which the "fish scale" pattern is visible. Flattened cells between the columnar cells and stellate reticulum-like area resemble the stratum intermedium of the tooth germ, which is believed to assist the ameloblast in producing enamel during odontogenesis (HE, 40×).

date[5,10].

AFS

Clinical characteristics: AFS is a very rare malignant odontogenic tumor, considered the malignant counterpart of AF, in which the ectomesenchymal component shows features of sarcoma, while the odontogenic epithelium remains bland[1]. To date, up to 100 cases of AFS have been reported in the English language literature. The mean age of the affected patients was 28 years (range: 3 to 89 years), with a slight predilection for male patients (male-to-female ratio: 1.6:1). AFS is more frequent in the mandible, and up to one-third of the cases have been derived from previously documented AF. Patients with AFS originating from AF have a mean age of 33 years, and those with *de novo* AFS (previous AF not demonstrated) are one decade younger. AFS usually appears as painful swelling, and paraesthesia may be present[5,25,26].

Radiographic features: As with most malignant intraosseous tumors, AFS presents as expansive ill-defined unilocular or multilocular radiolucency with bone destruction areas, perforation of cortical areas, irregular margins and occasional root resorption. It can be associated with unerupted teeth and eventually cause diffuse expansion and thinning of the cortex[2,27].

Histopathology: Histologically, AFS is similar to AF; however, the ectomesenchyme is hypercellular and malignant, while the epithelial component tends to decrease and virtually disappears in recurrent tumors (Figure 3A-C)[1,28]. Epithelial nests and cords remain inactive, presenting an immunohistochemical profile similar to AF and positivity for AE1/AE3[15-17] (Figure 3E). Proliferation-related markers such as Ki-67 and p53 can help distinguish AF and AFS, because they are virtually negative in the mesenchymal component and epithelium of AF and positive in a variable percentage in the malignant cells of AFS (Figure 3F).

Treatment and prognosis: Because AFS is locally very aggressive, with a high tendency to relapse, treatment includes wide surgical removal and long-term follow-up. Adjuvant radiotherapy has been used in some cases with favorable results[29], while the usefulness of chemotherapy has not been confirmed[26]. Recurrence occurs in approximately 20% and metastasis in only 4.5% of cases, but the mortality after 5 years of treatment is relatively high, estimated in 25.4% of cases[1,25,27].

Ameloblastic fibrodentinosarcoma/ameloblastic fibro-odontosarcoma

In the 2005 and 2017 WHO classifications of tumors, AFDS/AFOS are described together as tumors with histological features of AFS presenting dentinoid (fibrodentinosarcoma) or dentinoid and enameloid (fibro-odontosarcoma) (Figure 3D) [1,2].

Clinical and histopathological features: Clinically, AFDS/AFOS present as painful swelling of the jaws, with only 21 cases reported in the literature, as summarized in Table 2[9,30]. From these cases, 10 described enameloid formation, corresponding to AFOS. The age range of the reported cases was from 4 mo to 83 years, with a peak in the third decade. Approximately 40% of the cases recurred, two developed metastasis, and three patients died because of aggressive local invasion[31-33].

Immunohistochemically, AFDS/AFOS are similar to AFS, with odontogenic epithelium positive for AE1/AE3, facilitating the localization of epithelial nests in cases of mesenchymal predominance, excluding the diagnosis of other sarcomas. As discussed previously, proliferation markers such as Ki-67 and p53 confirm the aggressiveness of the lesion, helping to differentiate it from its respective benign counterpart [30,34,35].

Radiographic features: Radiographically, they appear as a uni- or multilocular radiolucency with variable dense opacities associated with impacted teeth. Irregular borders, expansion and perforation of the cortex are common, indicating a malignant tumor[34].

Treatment and prognosis: Treatment is based on wide local surgical excision, and long-term follow-up is advised[30]. We found 21 cases of AFDS/AFOS in the English language literature, 8 of which (38%) recurred, 2 developed distant metastasis, and 3 cases (15%) caused death.

Molecular characterization of mixed odontogenic tumors

To date, few studies have investigated the genetic/molecular profiling of mixed odontogenic tumors. Molecular testing (polymerase chain reaction followed by direct sequencing and next-generation sequencing) has revealed that 33% to 100% of benign mixed odontogenic tumors (AF, AFD, and AFO) and 71% of AFS harbor *BRAF* p.V600E mutation in their mesenchymal component (and rare cases in both components), unlike odontomas, which are BRAF wild-type. This finding suggests that a subset of AF, AFD and AFO differs molecularly from odontomas, likely supporting the distinct nature of these entities (neoplastic *vs* hamartomatous). The *BRAF* p.V600E mutation is involved in the pathogenesis of several tumors, including ameloblastoma, playing a role as a downstream activator of the MAPK signaling pathway, which regulates several cell processes, such as proliferation, survival and apoptosis[36-38].Confirming these findings, immunohistochemical reactions against BRAFV600E exhibited specific staining only in the stromal component, supporting the role of this mutation as a driver of the malignant stromal component[38]. Although the BRAF p.V600E mutation seems to be present in most AFSs in the study of Agaimy *et al*

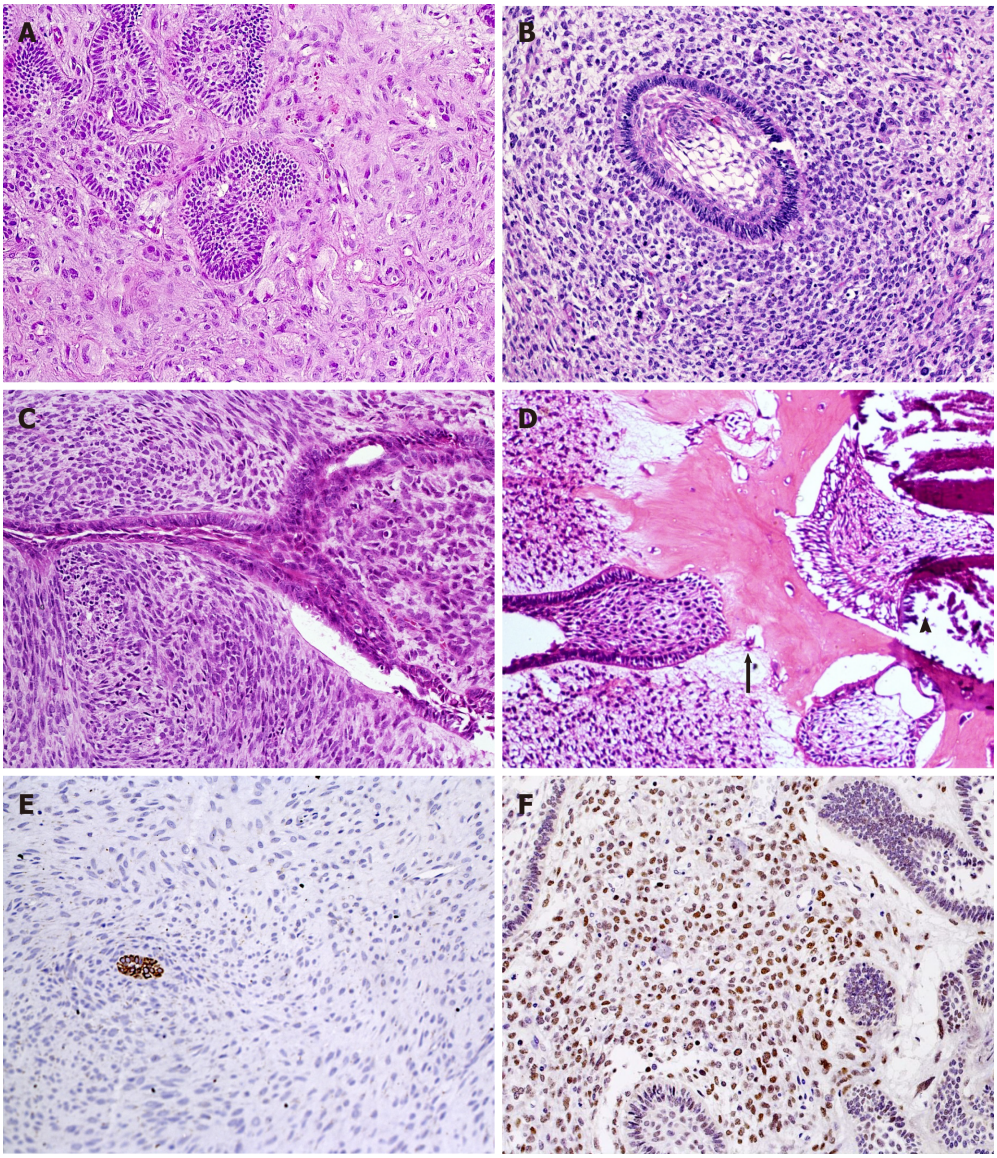

Figure 3 Histopathological aspects of ameloblastic fibromas and ameloblastic fibro-odontosarcoma. Marked pleomorphism and atypia in mesenchymal cells (HE, 20×) (A-C). A: Several mitotic figures, nuclear hyperchromatism and multinucleated and aberrant cells are seen in a highly pleomorphic sarcomatous component of ameloblastic fibromas, while epithelial islands remain benign; B: A follicular benign epithelial island is surrounded by hypercellularized sarcomatous proliferation; C: Malignant mesenchymal tissue resembling a storiform pattern and haphazard disposition of sarcomatous cells around an epithelial branching cord; D: Production of enamel matrix (arrowhead) and dentinoid (arrow) as well as malignant mesenchymal tissue (left side) are components of this ameloblastic fibro-odontosarcoma (A-D: HE, 20×); E: Cytokeratins can help to localize odontogenic epithelial cells within dominant sarcomatous proliferation (IHC for AE1/AE3, 20×); F: Most mesenchymal malignant cells show nuclear positivity for p53 antigen (IHC for p53, 20×).

[38], the NRAS p.Gln61Lys mutation was also detected in one AFS case, and another case was wild type.

DISCUSSION

AF, AFD and AFO present similar clinical, radiographic and microscopic features and were accepted as different entities until the 2017 WHO classification, which considers these entities as representing diverse stages of maturation of a developing odontoma, as suggested by Cahn and Blum in the past[39]. In summary, we suggest that the lesions referred to as AFD and AFO are more likely "developing odontomas" and are no longer considered mixed odontogenic tumors, as in the previous classifications of odontogenic tumors.

We consider that the WHO classification of these tumors in 2017 is unclear because it states that it is not possible to differentiate histologically between AF (true neoplasms) and early-stage odontomas before they differentiate and mature; therefore,

the existence of both lesions is accepted. However, no evidence has shown that AF matures to odontoma. If maturation of AF occurred, it would be expected that AF (immature lesion) would be diagnosed at an earlier age than AFO (mature lesion). However, an opposite trend has occurred: as the mean age of diagnosis of AFO is 9.6 years and that of AF is 14.9 years. However, because several AFOs have been reported in children in areas of odontogenesis, some cases might represent odontomas. Nevertheless, some cases of AFD/AFO arise in age groups that are not consistent with a hamartoma: from the 45 reviewed cases of AFD, 17 cases (37.7%) were aged ≥ 20 years, and 7 cases (15.5%) were aged ≥ 30 years, 4 of which were in the sixth and seventh decades of life.

The terms AFD and AFO were practically discarded from the latest WHO tumor classification, considering that once hard tissues are produced, these tumors are more likely to form odontomas[2,40]. Nevertheless, in this WHO classification, AFD and AFO might conceptually be neoplastic when reaching an exceptionally large size but without establishing a measure for this statement[2].

To avoid concepts that may be confusing and that are not appropriately supported by scientific evidence, we suggest not using the term developing odontomas and simply continuing to use odontomas if the clinical, radiographical and microscopic characteristics support this well-established diagnosis. Cases of a typical odontoma associated with AF could be termed AF associated with odontoma because odontomas can eventually be associated with other odontogenic tumors.

We accept that some cases are difficult to classify as AFO or odontoma because of the large amounts of hard dental tissues and because some cases of odontoma have been diagnosed as AFO. However, a cut off could be considered based on the proportion of hard and epithelial-ectomesenchymal tissues, as well as clinical (size, location, age, and clinical behavior) and radiographic features.

No evidence exists that all AF/AFD/AFO are "developing odontomas" because each of these tumors has its own clinicopathological features. AF is a well-recognized entity, and it should also be emphasized that no evidence is available that AF matures and forms small or large amounts of hard dental tissues, even in cases of recurrence.

AFD has no potential to produce enamel/enameloid; therefore, it cannot mature to an odontoma. However, some AFOs can produce large amounts of hard dental tissues and may mimic radiographically and microscopically odontomas; nevertheless, most AFOs present relatively few calcified areas. We reviewed 82 cases in the English literature with adequate radiographic documentation, most of which had small amounts of hard tissues: 59.8% presented few opacities or radiolucent images, 13.4% showed a higher number of scattered opacities, and only 26.8% presented a single opaque mass similar to odontoma. Even considering these cases rich and poor in calcified dental tissues diagnosed as AFO, evidence exists that cases poor in dental calcified structures evolve to those that mimic odontomas.

Recent molecular studies have shown genetic differences (principally, *BRAF*V600E mutation) between odontoma (BRAF wild type) and a subset of AF, AFD, AFO and most AFS, supporting that these lesions may represent distinct entities with a neoplastic nature[36-38].

In summary, we propose to continue to use the classical terms AFD and AFO because it is part of the 2017 WHO classification for malignant counterparts. This recommendation can be relevant for future clinical, microscopic and molecular studies to better clarify the subject and better understand the biology of these interesting odontogenic tumors.

Several aspects support the neoplastic nature of AF, AFD and AFO, such as their biological behavior, significant frequency of *BRAF* mutation, age of occurrence, amount of hard tissue and potential for malignant transformation into odontogenic sarcomas with or without the production of dental hard tissues. Among the 18 cases of AFDS/AFOS reported in the literature, 6 were related to a preexisting AFO, and this malignant transformation would not be expected in a hamartomatous lesion as a developing odontoma. The 2017 WHO classification accepts AFS, AFDS and AFOS as entities, and they can be de novo or derived from AF. This inconsistency in the nomenclature between benign and malignant corresponding tumors probably occurred because the topics of "odontogenic sarcomas" and "ameloblastic fibroma" were written by different authors in the 2017 WHO classification.

Odontogenic carcinosarcoma was added to the 2017 WHO classification based on 6 case reports, considering that it may arise de novo or can be derived from previous AF or AFS. However, we also found in the literature that, in two cases, ameloblastoma and malignant ameloblastoma were reported as the preceding tumors (Table 3). In contrast to AFS, in which metastasis is rare, 33% (3 cases) of odontogenic/ameloblastic carcinosarcomas presented biphasic metastasis (epithelial and sarcomatous

Table 3 Main data of 9 cases reported of ameloblastic/odontogenic carcinosarcoma in the literature

Case	Ref.	Sex/age	Location	Preexisting tumor	Progression
1	Tanaka *et al*[91]	M/63	Maxilla	Malignant ameloblastoma	Recurrence, metastasis and death
2	Slama *et al*[92][1]	F/26	Mandible	AF	Metastasis and death
3	Kunkel *et al*[3]	M/52	Mandible	No	Recurrence, metastasis and death
4	DeLair *et al*[93]	F/19	Mandible	AF	No recurrence
5	Chikosi *et al*[94]	F/9	Mandible	Ameloblastoma	Recurrence and death
6	Kim *et al*[4]	M/61	Mandible	No	No recurrence
7	Dos Santos *et al*[95]	M/42	Maxilla	No	Unknown
8	Soares *et al*[96]	M/22	Mandible	No	No recurrence
9	Soares *et al*[96]	F/19	Mandible	Rhabdomyosarcoma (parotid region)[2]	Post-surgical systemic infection and death

[1]Article in French, abstract in English.
[2]Thirteen years before, treated with surgical resection followed by radiotherapy.
M: Male; F: Female; AF: Ameloblastic fibroma.

components), and 5 of 9 cases resulted in death[3]. Thus, this entity was recently recognized at the present WHO classification. Immunohistochemically, positivity for p53 and a Ki-67 index > 45% in both carcinomatous and sarcomatous components can be useful to confirm the diagnosis[2].

It is reasonable to consider that basic benign and malignant neoplasms are AF and AFS and that the presence of small amounts of dental hard tissues does not significantly alter the biological characteristics and clinical behaviors of these entities [1,13]. Although not clearly established, the presence and higher amount of hard tissues may indicate less aggressiveness and possibly lower potential of malignant transformation. In this context, AFO should have a better prognosis than AF/AFD, with a lesser tendency for malignant transformation. AFDS/AFOS seem to have a similar rate of recurrence as AF; however, the metastasis and mortality indexes seem to be higher in AFSs. Additionally, the number of cases of AFD/AFO and AFDS/AFOS reported is very small, making comparisons of these tumors with AF/AFS difficult.

Reports of AFSs have been present for several years, possibly as AF/AFO/AFD that have suddenly followed an aggressive course before being treated, indicating a possible malignant transformation[25,41].

CONCLUSION

In summary, we reviewed the principal clinical, histopathological and molecular characteristics of AF, AFD and AFO and their malignant counterparts. Odontogenic/ameloblastic carcinosarcoma was cited because, according to reports, it can arise from preexisting AF. We consider that the recent 2017 WHO classification does not clarify the subject when considering AFD and AFO as developing odontomas. According to the clinical, radiographical, histopathological and molecular features of the cases reviewed, we suggest that AFD and AFO should continue to be considered benign neoplasms. Thus, the nomenclature of these mixed benign odontogenic tumors would be congruent with the classification of ameloblastic/odontogenic sarcomas. Additionally, further studies are warranted to compare these interesting odontogenic tumors and finally better clarify and understand their similarities and differences.

ARTICLE HIGHLIGHTS

Research background

Ameloblastic fibromas and ameloblastic fibrosarcomas are rare odontogenic tumors, and controversy exists in the classification of cases presenting hard-tissue production: Ameloblastic fibrodentinoma (AFD) and ameloblastic fibro-odontoma (AFO). These

cases are currently considered "developing odontomas" (hamartomatous lesions). There is still controversy as to whether they are true hamartomas or neoplasms.

Research motivation

The authors consider that the recent 2017 WHO classification does not clarify the subject when considering AFD and AFO as "developing odontomas". According to the clinical, radiographical, histopathological and molecular features of the cases reviewed, we suggest that AFD and AFO should continue to be considered benign neoplasms.

Research objectives

The objective was to analyze the clinicopathologic features of these lesions and discuss the changes in the 2017 WHO classification.

Research methods

For this systematic review an electronic literature search was performed in the PubMed/MEDLINE database. An exhaustive search was made of all the existing information on these mixed odontogenic tumors.

Research results

Several aspects of AFO and AFD, such as biological behavior, age of occurrence, amount of hard tissue, and potential for malignant transformation into odontogenic sarcomas, support the neoplastic nature in most of the reported cases.

Research conclusions

Considering the clinical, radiographic, histopathological, and molecular characteristics of odontogenic lesions with hard tissue production, we suggest that these types of lesions should continue to be recognized as odontogenic tumors by maintaining the classically used terms. This recommendation will be relevant for future clinical, microscopic, and molecular studies to better understand the biology of these interesting odontogenic tumors. This new information will be relevant for the clinical conduct to be followed in these tumors.

Research perspectives

Future research should be focused on the comparative molecular study between these odontogenic neoplasms and odontomas; trying to clarify molecular differences between neoplasia and hamartoma.

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SYSTEMATIC REVIEWS

Carcinosarcoma of gallbladder: A world review

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Abstract

BACKGROUND

Gallbladder carcinosarcoma is a rare hepatobiliary tumor comprising of both carcinomatous and sarcomatous components. Due to its rarity, the literature with regards to the topic is scarce and currently lacking, spanning less than 100 cases.

AIM

To summarize the current literature on gallbladder carcinosarcoma.

METHODS

A literature review was performed on the PubMed database using the keywords "Gallbladder" AND "Carcinosarcoma" from 1970 to 2021. Additionally, similar searches were performed on MEDLINE and Web of Science.

RESULTS

Risk factors noted include female gender, gallstones and chronic cholecystitis. In the absence of any diagnostic biochemical testing or tumor markers, imaging modality serves as the key initial impression tool, which can be histologically confirmed only post-resection. While surgery is the only curative option, the use of adjunctive chemotherapy has been considered on top of excision in recent years, with some success.

CONCLUSION

While this study has taken steps to bridge the gap in the literature, more cases should be reported to further ascertain the current associations and management potential for gallbladder carcinosarcoma.

Key Words: Carcinosarcoma; Gallbladder; Gallstone; Malignancy; Carcinoma; Sarcoma

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Core Tip: Gallbladder carcinosarcoma (GBCS) while rare, is an important histological subtype of gallbladder malignancy as it is associated with poor prognosis. Most GBCS patients tend to present late. As of now, the primary method of diagnosis is that of a pathological analysis with the main stay of treatment being surgical excision. Furthermore, the clinical diagnosis of GBCS remains extremely challenging given its seemingly nonspecific clinical features. We aim to provide an in-depth world review of the known cases of GBCS in order to identify unifying features of the disease and to assess effective management strategies that have been employed by clinicians.

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INTRODUCTION

Gallbladder carcinosarcoma (GBCS) is defined by the presence of both carcinomatous and sarcomatous components in the tumor, making it a rarity even amongst the uncommon gallbladder cancer family[1]. While its history is deep-rooted, with the first case being reported by Karl[2] in 1907, less than 100 cases have been reported since. In 2008, Zhang *et al*[3] sought to collectively analyze the 70 cases in the literature at that time. However, Zhang *et al*[3] noted the need for a larger scale case series to provide more information on the neoplasm for better accuracy and reliability. Since then, there has been a gap in the literature for such an analysis (Figure 1). This study aims to fill this gap by providing a comprehensive overview of GBCS.

MATERIALS AND METHODS

A literature review was performed on the PubMed database using the keywords "Gallbladder" AND "Carcinosarcoma" from 1970 to 2021. Additionally, similar searches were performed on Medline and Web of Science. The last search was performed on January 31, 2021. After removing duplicate results from similar databases, the search yielded 105 articles: 16 non-English and non-Japanese studies and 12 unrelated topics (animal studies, gallbladder carcinoma and non-gallbladder pathology) were excluded. Out of the remaining 77 articles, seven were not case reports or case series on GBCS and thus excluded. The remaining 70 articles including 76 patients were included in the final analysis (Table 1)[1,4-72]. Article filtering and exclusion was done according to PRISMA guidelines (Figure 2). Data extracted included study year, age and gender of the patient, clinical presentation, risk factors, laboratory investigations, tumor markers, the ultrasound imaging findings, location of the lesion within the gallbladder, size of the lesion, initial diagnosis, method of confirming the diagnosis, immunohistochemical results (vimentin, cytokeratin, Ki-67), management and prognosis of the patient. Kaplan-Meier survival curves were compared between lesions larger than 5 cm and those smaller than 5 cm as data by Zhang *et al*[3] suggested that tumors smaller than 5 cm had better survival. For all statistical tests, a P value of 0.05 was used to determine statistical significance.

RESULTS

Seventy-eight patients with a mean age of 66.4 years (range: 40-91 years) were reported during the study period. The patients were predominantly female (n = 55, 72.4%) with a gender ratio of 2.62. Nine patients (11.8%) had chronic cholecystitis, and 1 patient each had hepatitis C and abnormal pancreaticobiliary maljunction (APBJ). Of those who reported the presence of gallstones, a majority noted the presence of gallstones (n = 35/42, 83.3%). The majority of patients complained of abdominal pain (n = 58, 76.3%), most of which was localized to the right upper quadrant. Twenty-two patients (28.9%) presented with constitutional symptoms (either unexplained loss of

Table 1 Summary of 76 reported cases of gallbladder carcinosarcoma from 1970 to 2021

No.	Year	Ref.	Age/sex	Risk factors for GB CA (stones)	Clinical presentation	Liver function tests	Position of CA	Tumor markers (CEA, AFP, CA 19-9)	Size (mm)	Initial diagnosis	Confirmatory diagnosis (mode)	Stage (UICC)	Survival (mo)	IHC positives	Further management
1	2020	Khurram *et al* [4]	64/F	No stones	RUQ pain, intermittent fever, abdominal distension	AST, GGT elevated	Fundus	Normal	132 × 97 × 110	Hepatic abscess	Cholecystectomy	NA	NIL mentioned	CK	NA
2	2020	Ayoub *et al*[5]	66/M	NA	RUQ pain	Normal	Body	Normal	150 × 80 × 60	Gallbladder malignancy	Cholecystectomy and lymphadenectomy	IVA	12+	Vimentin, CK	NA
3	2020	Kaneko *et al*[6]	70/F	NA	Obstructive jaundice	NA	NA	Normal	110 × 70 × 34	Gallbladder malignancy	Cholecystectomy	NA	44+	CK, Ki-67	NA
4	2020	Siddiqui *et al*[7]	57/M	NA	Abdominal pain, nausea, LOW, LOA	ALP, total bilirubin elevated	Fundus	NA	620	Gallbladder malignancy	ERCP (unsuccessful), PTC with internal-external biliary drainage catheter	NA	NA	Vimentin	NA
5	2020	Mochizuki *et al* [8]	88/F	Gallstones	Chills, tremors, vomiting	NA	Body	NA	60 × 25	Acute cholecystitis	Cholecystectomy	NA	10 +	Ki-67	NA
6	2019	Varshney *et al* [9]	50/M	Gallstones	RUQ pain, obstructive jaundice	AST, ALT, bilirubin elevated	Fundus	Normal	65 × 55	Gallbladder malignancy	radical cholecystectomy with standard lymphadenectomy	NA	6+	Vimentin, CK	Adjuvant chemotherapy
7	2019	Aldossary *et al* [10]	40/M	Gallstones	RUQ pain	Normal	Entire gallbladder	Normal	115 × 92 × 50	Gallbladder malignancy	Open lap, radical cholecystectomy, extended R hemi w IC anastomosis, liver resection	IVB	6	Vimentin	Adjuvant chemotherapy
8	2019	Aldossary *et al* [10]	52/F	No stones	RUQ pain	ALT, AST elevated	Fundus	CA19-9 level of 154.3 IU/mL, with normal levels of AFP and CEA	136 × 120 × 95	Gallbladder Malignancy	Open lap, radical CCY, transverse chole, Roux en Y + distal gastrectomy	IVB	3	Vimentin, CK	NA
9	2019	Aldossary *et al* [10]	62/F	Gallstones	RUQ pain, nausea, anorexia	Normal	Body	Normal	27 × 9	Gallbladder malignancy	Lap CCY	II	86+	Vimentin, CK	Adjuvant chemotherapy

10	2019	Alratroot *et al* [11]	52/F	Xanthogranulomatous cholecystitis	RUQ pain	GGT elevated	Fundus	CA 19-9 154.33 IU/mL	110 × 60	Gallbladder malignancy	Laparotomy with radical cholecystectomy, transverse colectomy, distal gastrectomy, omentectomy and liver bed resection	III	1.5+	Vimentin, CK	Adjuvant chemotherapy
11	2019	Matsubayashi *et al*[12]	72/F	Pancreaticobiliary maljunction	RUQ pain	ALP, GGT elevated	Entire gallbladder	Normal	90 × 85	Gallbladder malignancy	Laparotomy and extended cholecystectomy	IIIA	73+	Vimentin, CK	NA
12	2018	Doniparthi *et al* [13]	49/M	NA	Epigastric pain	AST, ALT, lipase elevated	NA	Normal	32	Acute cholecystitis	Lap cholecystectomy, followed up by robotic liver resection and lymphadenectomy	NA	NA	NA	NA
13	2018	Koustav *et al* [14]	40/F	NA	RUQ pain		NA	CA19-9 elevated	43 × 51	Gallbladder malignancy	Staging laparoscopy + extended cholecystectomy	NA	NA	NA	NA
14	2018	Trautman *et al* [15]	73/F	Chronic cholecystitis	Abdominal distension, constipation, vomiting, LOW	AST, ALT, ALP elevated	NA	Beta-HCG elevated		Gallbladder malignancy	Diagnostic laparoscopy	NA	0.5	Vimentin	Palliative (NM)
15	2017	Furuya *et al*[16]	61/F	NA	RUQ pain	Normal	NA	Normal	15 × 15	Chronic cholecystitis with stone	Cholecystectomy	NA	NA	NA	NA
16	2016	Hu *et al*[17]	68/F	Cholelithiasis	RUQ pain, fever	Normal	Body	CA19-9 elevated	16 × 15 × 13	Gallbladder malignancy	Cholecystectomy	NA	1	NA	NA
17	2016	Cruz *et al*[18]	52/F	Gallstones	RUQ pain	ALT AST elevated	Entire gallbladder	Normal	170 × 125	Gallbladder malignancy	Cholecystectomy	NA	1	Vimentin, CK	Palliative (NM)
18	2016	Dong *et al*[19]	61/M	NA	Abdominal distension	NA	NA	Normal	180	Gallbladder malignancy	Resection (not specified)	NA	NIL mentioned	Ki-67	NA
19	2016	Gupta *et al*[20]	46/F	NA	RUQ pain	NA	Fundus	All normal	350 × 250 × 200	Gallbladder malignancy	Radical cholecystectomy with hepato-duodenal ligament lymph node clearance and segment 4b/5 liver resection	NA	15 (still alive)	Vimentin, CK	Adjuvant chemotherapy
20	2016	Wong *et al*[21]	52/F	NA	Abdominal pain	NA	Entire gallbladder	CA19-9 elevated	75	NA	Autopsy	III	6	Vimentin, CK	Adjuvant chemotherapy
21	2016	Ansari *et al*[22]	50/F	NA	RUQ pain	Normal	Entire gallbladder	Normal	50 × 40	NA	Radical cholecystectomy	II	13 mo (still alive)	Vimentin, CK, Ki-67	Adjuvant chemotherapy
22	2015	Gao *et al*[23]	62/M	Chronic cholecystitis	RUQ pain	Normal	Entire gallbladder	Normal	50 × 40	Gallbladder malignancy	Simple cholecystectomy	II	0	Vimentin, CK	NA
23	2015	Tonouchi *et al* [24]	87/M	No stones	Abdominal pain	NA	NA	NA	60 × 55	Diffuse peritonitis	Cholecystectomy with partial transverse colectomy around the fistula	NA	Lost to follow-up	Vimentin, CK	NA

24	2015	Faujdar *et al* [25]	60/F	NA	RUQ pain, fever	Normal	Entire gallbladder		120 ×70 × 60	Gallbladder malignancy	Cholecystectomy	NA	60+	Vimentin, CK	NA
25	2014	Wada *et al*[26]	68/M	NA	Right flank pain	GGT elevated	NA	Normal	85 × 70	Gallbladder malignancy	Extended right hepatectomy with portal thrombectomy with hepatoduodenal ligament lymphadenectomy	NA	51+	Vimentin, CK, Ki-67	Adjuvant chemotherapy
26	2014	Kishino *et al* [27]	70s/F	NA	Referred for suspected GB cancer (presenting complaint not mentioned)	NA	Fundus	NA	68	Gallbladder malignancy	Cholecystectomy	NA	1.5+	Vimentin, CK	NA
27	2013	Wang *et al*[28]	68/F	Chronic cholecystitis, cholecystolithiasis	RUQ pain, jaundice, fever	ALT, ALP elevated	NA	CEA, CA19-9, AFP elevated	100 × 70 × 50	Gallbladder malignancy	Cholecystectomy with liver segmentectomy (S4a+S5) and a lymph node dissection, followed by resection of the extrahepatic bile duct and a Roux-en-Y type hepatic cholangiojejunostomy	NA	6+	Vimentin, CK	NA
28	2013	Khanna[29]	45/F	NA	RUQ pain	Normal	Body		60 × 40	Gallbladder malignancy	Laparotomy and simple cholecystectomy with wedge resection	NA	3	Vimentin, CK, Ki-67	NA
29	2013	Li *et al*[30]	64/M	Chronic cholecystitis	RUQ pain	NA	NA	CEA, CA19-9 elevated	40 × 30 × 30	NA	Cholecystectomy, R hemicolectomy, resection of multiple hepatic metastases	NA	3+	Vimentin, CK, Ki-67	NA
30	2012	Kim *et al*[31]	72/F	Gallstones	Abdominal pain	Normal	Fundus	Normal	65 × 45 × 45	Gallbladder malignancy	Radical cholecystectomy with wedge resection of liver combined with hepatoduodenal ligament lymphadenectomy	NA	4	NA	Adjuvant chemotherapy
31	2012	Kim *et al*[31]	81/M	NA	Epigastric pain	Normal	Fundus	Normal		Gallbladder malignancy	Cholecystectomy with liver segmentectomy (S4a,5) and lymph node dissection	NA	13	Vimentin, CK	NA
32	2012	Sadamori *et al* [32]	80/M	NA	RUQ pain, fever		Entire gallbladder		76 × 27	Gallbladder malignancy	Cholecystectomy with liver segmentectomy (S4a and S5) and lymph node dissection	NA	2+		Adjuvant chemotherapy
33	2012	Kataria *et al*[33]	55/F	NA	RUQ pain	Normal	Fundus	Normal	70 × 50 × 30	NA	Cholecystectomy, wedge resection of liver with resection of transverse colon and paraduodenal lymph node	NA	6	Vimentin, CK	NA
34	2012	Parreira *et al* [34]	59/F	NA	RUQ pain	Normal	NA	NA	NA	NA	Conventional cholecystectomy	NA	2	NA	NA

35	2012	Park *et al*[35]	77/F	NA	RUQ pain	AST, ALT elevated	NA	CA19-9, CA-125 elevated	78 × 55 × 12	Gallbladder malignancy	Laparotomy, followed by cholecystectomy and lymph node dissection	IIIB	10+	NA	NA
36	2012	Ishida *et al*[36]	62/F	NA	Incidental finding on radiograph for left calcaneal fracture	Normal	Entire gallbladder	Normal	52 × 38	Gallbladder malignancy	Open cholecystectomy	NA	8	NA	NA
37	2011	Lee *et al*[37]	77/F	No stones	RUQ pain	Not mentioned	Body	CA19-9, CA-125 elevated	80 × 70 × 30	Gallbladder malignancy	Cholecystectomy	NA	1.5+	Vimentin, CK	NA
38	2011	Pu *et al*[38]	59/F	Cholecystolithiasis	RUQ pain, fever	Normal	Body	CA19-9 elevated	120 × 25 × 60	Gallbladder malignancy	Exploratory laparotomy, followed by radical LN resection and hepatocholangojejunostomy Roux-En-Y	II	0	CK	Adjuvant chemotherapy
39	2011	Krishnamurthy *et al*[39]	83/M	No stones	Abdominal pain	NA	NA	NA	NA	NA	Laparoscopic cholecystectomy	NA	48+	Vimentin, CK	NA
40	2009	Kohtani *et al* [40]	84/M	Chronic cholecystitis	RUQ pain	Serum glutamic oxaloacetic transaminase, GGT elevated	Neck	NA		Gallbladder malignancy	Open cholecystectomy	II	3+	Vimentin, CK, Ki-67	Adjuvant chemotherapy
41	2009	Agarwal *et al* [41]	60/F	NA	RUQ pain, fever	Normal	Neck	NA	70 × 50 × 40	Gallbladder malignancy	Staging laparoscopy, laparotomy, simplex cholecystectomy	NA	24+	Vimentin, CK	NA
42	2009	Magata *et al*[42]	78/F	NA	RUQ pain	NA	Body	CEA elevated	115 x 40 x 35	Gallbladder malignancy	Whole-layer cholecystectomy with regional lymph node dissection	NA	6+	Vimentin, CK	NA
43	2009	Shimada *et al* [43]	69/M	Choledocholithiasis	Fever	Normal	Entire gallbladder	AFP elevated	90 × 50	Gallbladder malignancy	Laparotomy, cholecystectomy, lymph node dissection	NA	54+	Vimentin, CK, Ki-67	NA
44	2009	Uzun *et al*[44]	70/M	NA	RUQ pain	Normal	Fundus	Normal	100 × 60 × 30	Gallbladder malignancy	Radical cholecystectomy, wedge resection of liver-gallbladder bed with hepatoduodenal ligament lymphadenectomy	NA	8	CK, Ki-67	NA
45	2006	Kubota *et al*[45]	72/M	NA	RUQ pain, fever	AST, ALT, ALP elevated	NA	Normal	70 × 55 × 40	Gallbladder malignancy	En bloc resection of the gallbladder and segments 4a and 5 of the liver, partial colectomy, and lymph node dissection	NA	6	NA	NA
46	2005	Akatsu *et al*[46]	76/F	Gallstones	Incidental	Normal	NA	Normal		Gallbladder	Extended cholecystectomy,	NA	2	Vimentin,	NA

					finding on follow-up for cholelithiasis					malignancy	liver 4b and 5 resection			CK	
47	2005	Huguet *et al* [47]	64/F	Cholecystitis	RUQ pain, fever	Normal	Entire gallbladder	Normal	120 × 100 × 70	Gallbladder malignancy	A cholecystectomy with wedge resection of the gallbladder fossa (involving liver segments 4 and 5), extrahepatic bile duct excision, non–pylorus-preserving pancreaticoduodenectomy with excision of 15 cm of proximal jejunum, and right hemicolectomy	NA	60	Vimentin, CK	NA
48	2005	Sodergren *et al* [48]	64/F	NA	Malaise and LOA	ALP Elevated	NA	NA	20 × 12 × 12	NA	Extrahepatic radical bile duct resection with hepatic and coeliac lymph node clearance followed by right hepaticodochojejunostomy to a jejunal Roux loop	NA	5	Vimentin, CK	NA
49	2005	Sodergren *et al* [48]	60/F	NA	Painless jaundice	NA	NA	NA	90	Gallbladder malignancy	Cholecystectomy and extrahepatic bile duct resection with lymph node clearance	NA	2	Vimentin, CK	Palliative (NM)
50	2004	Takahashi *et al* [49]	84/F	NA	RUQ pain	NA	Body	CEA, CA19-9 elevated	84 × 40 × 30	Gallbladder malignancy	Cholecystectomy and transverse colon partial colectomy	NA	2	Vimentin, CK	
51	2003	Kim *et al*[50]	61/F	No stones	RUQ pain	Normal	Neck	Normal	45 × 40 × 40	Gallbladder malignancy	Cholecystectomy with common bile duct resection	NA	2	Vimentin	Palliative (NM)
52	2002	Al-Sheneber *et al*[1]	68/F	Acute cholecystitis, gallstones	RUQ pain	Normal	NA	CEA elevated	148 × 80	Gallbladder malignancy	CT guided needle biopsy of the upper abdominal mass	NA	7	Vimentin, CK	NA
53	2002	Hotta *et al*[51]	53/M	Chronic cholecystitis, gallstones	RUQ Pain	Normal	NA	Normal	1100	Gallbladder malignancy	Cholecystectomy with resection of subsegmentectomy of liver S5 and a resection of transverse colon at the second operation	II	2	NA	Adjuvant chemotherapy
54	2002	Ajiki *et al*[52]	69/F	Gallstones, left renal tumor	Epigastric pain	Normal	NA	CA19-9 elevated	NA	Double cancers of the left kidney and gallbladder	Left renal excision, cholecystectomy with liver segmentectomy (S4a, S5), and lymph node dissection	NA	NA	NA	Adjuvant chemotherapy
55	2000	Yavuz *et al*[53]	50/F	NA	RUQ pain	NA	Body	NA	80 × 60 × 60		Exploratory laparotomy -> cholecystectomy, liver wedge biopsy	NA	NA	NA	NA

56	1999	Eriguchi *et al* [54]	65/F	Gallstones	RUQ pain	Normal	Entire gallbladder	NA		Gallbladder malignancy	Cholecystectomy	I	16+	NA	NA
57	1997	Ryś *et al*[55]	67/F	Gallstones	Abdominal pain, LOW	NA	Fundus	NA	10 × 15	Gallbladder malignancy	Hemicolectomy and cholecystectomy	NA	2	Vimentin	NA
58	1996	Nakagawa *et al* [56]	60/F	NA	Abdominal pain, fever	Normal	Body	NA	70 × 40	Gallbladder malignancy	Mass reduction surgery	NA	NA	NA	NA
59	1994	Fagot *et al*[57]	83/F	Gallstones	Vomiting, fever, right RHC pain	Total bilirubin elevated	Fundus	NA	45	NA	Surgery (not defined)	NA	12+	NA	NA
60	1992	Nakazawa *et al* [58]	63/F	NA	Nausea	Normal	Body	Normal	30 × 30	Gallbladder malignancy	Pancreaticoduodenectomy	NA	NA	NA	NA
61	1990	Ishihara *et al* [59]	58/F	NA	Abdominal pain	NA	Fundus	NA	50 × 80	Gallbladder malignancy	Cholecystectomy	NA	11+	Vimentin	NA
62	1988	Lumsden *et al* [60]	81/F	Gallstones	RUQ pain, LOW, LOA	Total bilirubin, ALP, GOT elevated	NA	NA	50 × 20 × 20	Biliary neoplasm	Cholecystectomy	NA	12+	NA	NA
63	1987	Hasegawa *et al* [61]	61/M	NA	RUQ pain	Normal	Entire gallbladder	NA	150	Gallbladder malignancy	Resection (not specified)	NA	6	NA	NA
64	1987	Herrera-Goepfert *et al* [62]	60/F	Gallstones	Abdominal pain, jaundice, LOW		Entire gallbladder	NA	70 × 40	Gallbladder malignancy	Autopsy	NA	NA	NA	NA
65	1986	Inoshita *et al* [63]	53/M	Gallstones	RUQ pain, jaundice	Total bilirubin, ALP, GOT, GPT elevated	Neck	NA	NA	Choledocholithiasis	Open laparotomy	NA	17	NA	NA
66	1985	Lopez *et al*[64]	78/F	No stones	Anorexia, LOW	Normal	NA	NA	NA	Gallbladder empyema	Open laparotomy	NA	NA	NA	NA
67	1984	Born *et al*[65]	90/F	Gallstones	Anorexia, nausea, vomiting	Amylase elevated	NA	NA	150 × 150 × 10	NA	Exploratory laparotomy	NA	3	NA	NA
68	1982	von Kuster *et al* [66]	91/F	Gallstones	RUQ pain, fever	GOT, ALP elevated	NA	NA	20	Gallbladder empyema	Exploratory laparotomy	NA	0	NA	NA
69			77/F	NA	Bleeding in GI tract (lower)	Normal	NA	NA	30	NA	Exploratory laparotomy	III	31+	NA	NA
70	1982	Aldovini *et al* [67]	75/F	Gallstones	Abdominal pain	ALP, SGT elevated	NA	NA	90	NA	Cholecystectomy	NA	8+	NA	NA
71	1982	Yamagiwa *et al* [68]	78/F	NA	RUQ pain	NA	NA	NA	NA	NA	NA	NA		NA	NA

72	1980	Mansori *et al* [69]	81/M	Gallstones	Abdominal pain	GOT, ALP elevated	NA	NA	NA	NA	Exploratory laparotomy	NA	0.5	NA	NA
73	1973	Higgs *et al*[70]	77/M	Gallstones	Jaundice	ALP, GOT elevated	NA	NA	NA	NA	Cholecystectomy and CBDE	NA	1	NA	NA
74	1971	Mehrotra *et al* [71]	45/F	Gallstones	RUQ pain	Normal	Neck	NA	50 × 40 × 30	NA	Open laparotomy	NA	4	NA	NA
75	1970	Appelman *et al* [72]	91/M	Gallstones, chronic cholecystitis	Obstructive jaundice	AST, ALT, ALP elevated	Fundus	NA	NA	Pancreatic cancer	Autopsy	NA	0.5	NA	NA
76	1970	Appelman *et al* [72]	75/F	Gallstones, chronic cholecystitis	RUQ pain	ALP elevated	Fundus	NA	50 × 50 × 20	NA	Cholecystectomy	NA	1	NA	NA

GBCA: Gallbladder cancer; CEA: Carcinoembryonic antigen; AFP: Alpha-fetoprotein; CA 19-9: Carbohydrate antigen 19-9; UICC: Union for International Cancer Control; IHC: Immunohistochemistry; RUQ: Right upper quadrant; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl transferase; ERCP: Endoscopic retrograde cholangiopancreatography; PTC: Percutaneous transhepatic cholangiogram; CCY: Cholecystectomy; NA: Not available; NM: Not mentioned; NIL: None; CR: Complete response; LOA: Loss of appetite; LOW: Loss of weight; R hemi w C: Right hemihepatectomy with cholecystectomy; M: Male; F: Female; Lap: Laparoscopic; Chole: Cholecystectomy; LN: Lymph node; CT: Computerized tomography; RH: Right hepatectomy; GI: Gastrointestinal; CBDE: Common bile duct exploration; HCG: Human chorionic gonadotropin; SGOT: Serum glutamic-oxaloacetic transaminase; SGPT: Serum glutamic-pyruvic transaminase.

weight, anorexia or lethargy). Nineteen patients (25.0%) had nausea and vomiting, and 13 patients (17.1%) were febrile. Two patients (2.6%) were asymptomatic when diagnosed.

Liver function test was the common serum biochemical test reported (n = 57). Deranged liver function tests were reported in 25 (43.9%) patients. Tumor markers were variably reported. The following tumor markers were elevated: carbohydrate antigen 19-9 (CA19-9) (n = 9/27, 33.3%), carcinoembryonic antigen (n = 5/27, 18.5%) and alpha-fetoprotein (n = 2/12, 16.6%) in some patients. Also, CA-125 was elevated in 2 patients.

Forty-three patients had the location of the gallbladder tumor reported. Fundus was the most common location (n = 15, 34.9%), followed by body (n = 10, 23.3%) and neck (n = 5, 11.6%). In 14 patients (32.5%), the tumor filled the entire gallbladder lumen, and thus exact position could not be determined. Fifty-nine patients had initial diagnosis reported. Out of these 59 patients, gallbladder malignancy was the primary diagnosis in the majority of patients (n = 49, 83.1%). Ten patients (16.9%) were initially diagnosed with other pathologies: cholelithiasis (n = 1), cholecystitis (n = 3), gallbladder empyema (n = 2), diffuse peritonitis (n = 1), pancreatic cancer (n = 1), biliary neoplasm (n = 1) and pyogenic liver abscess (n = 1).

Confirmation of diagnosis was reported in all but 1 patient (n = 75). It was mostly done *via* surgical resection, either diagnostic cholecystectomy or laparotomy (n = 70, 93.3%). In the remaining 5 patients, diagnosis was made by fluid analysis from percutaneous cholecystostomy (n = 1, 1.3%), computerized tomography (CT) scan guided needle biopsy (n = 1, 1.3%) and autopsy (n = 3, 4.0%). Staging of the cancer was

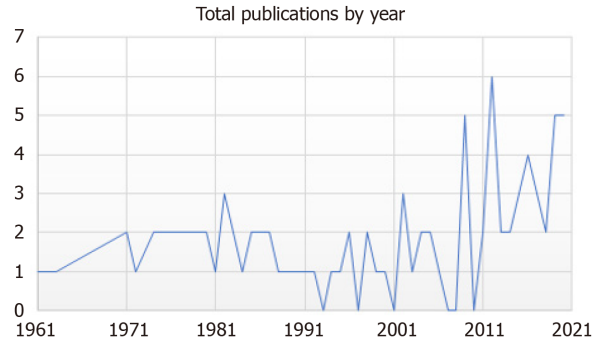

Figure 1 Paucity of gallbladder carcinosarcoma reports and trends by decade.

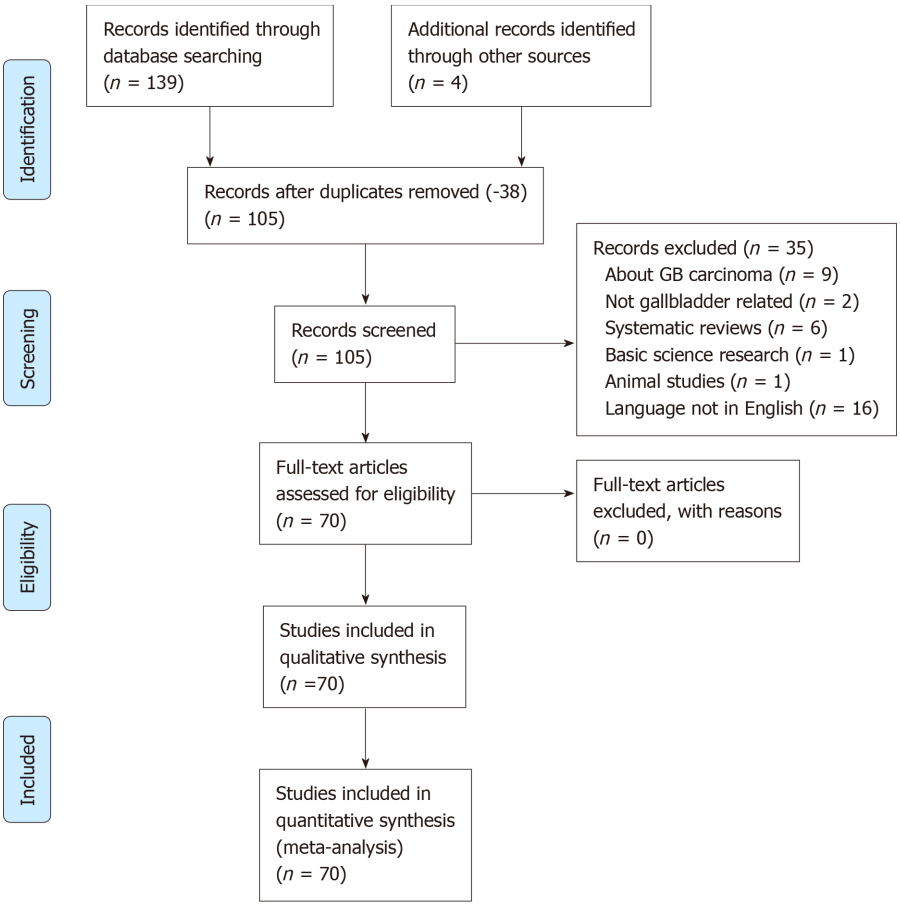

Figure 2 PRISMA diagram of articles searched on gallbladder carcinosarcoma. GB: Gallbladder

reported infrequently, with TNM system being the most common (n = 15, 19.7%). The majority of patients had stage II (n = 6, 40.0%) and stage III disease (n = 5, 33.3%). Three patients had stage IV disease (20.0%), and 1 patient had stage I disease (6.67%). Immunohistochemical stains (vimentin for mesenchymal components and cytokeratin for epithelial components) were reported in 50 patients (68.5%). Vimentin (n = 42, 84.0%), cytokeratin (n = 39, 78.0%) and Ki-67 staining (n = 7, 14.0%) were variably positive.

Fourteen patients (18.4%) received adjuvant chemotherapy. Various chemotherapy combinations included: gemcitabine and cisplatin, leucovorin and 5-fluorouracil (5-FU), cisplatin and doxorubicin, cisplatin and 5-FU, tegafur-uracil and gemcitabine and oxaliplatin and 5-FU. Palliative treatment was chosen in 4 patients (5.26%). Amongst all those reported, 32 patients contained both survival and tumor size data. Kaplan-Meier survival analysis was performed (Figure 3), and there was no significant difference in survival times (P = 0.301) for patients with tumors less than 5 cm in diameter compared to those with larger tumors.

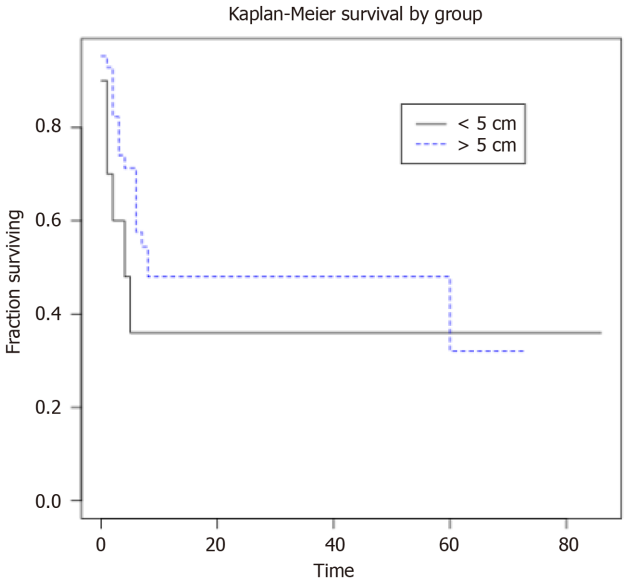

Figure 3 Kaplan-Meier survival curve measuring the difference in survival between patients with gallbladder carcinosarcoma of less than 5 cm diameter and more than 5 cm diameter (*P* = 0.301).

DISCUSSION

Gallbladder cancer is a rare neoplasm, accounting for about 0.5% of all gastrointestinal malignancies[73]. Most common gallbladder cancer is adenocarcinoma. GBCS is a rare form of gallbladder cancer, with only 78 cases reported. GBCS is characterized by carcinomatous and sarcomatous components and is made up of both epithelial and mesenchymal components. Commonly, the epithelial component consists of adenocarcinoma followed by the less common squamous cell carcinoma[74]. While there are multiple theories to justify the mixture of the epithelial and mesenchymal components, there is no consensus on the pathophysiology of the neoplasm. GBCS is considered the most aggressive biliary tract malignancy, usually discovered at late stages, and has poor prognosis[3].

Incidence

In terms of patient demographics, our results are consistent with the report of Zhang *et al*[3]. In a report including 68 GBCS patients, those authors reported a median age of 68 years (range: 45 to 91 years) with female predominance (female:male = 2.7:1), consistent with our results with a gender ratio of 2.32 and a mean age of 66.0 years (range: 40-91 years). Female preponderance is likely due to increased prevalence of gallstones in females. Zhang *et al*[3] noted gallstones in 66.7% of their patients. In our study, the incidence of gallstones was high (83%). However, gallstone presence was not specific nor sensitive in the diagnosis of GBCS, as not only are they a common finding in cancers of the gallbladder, only 1%-5% of patients with gallstones develop gallbladder malignancies. In our analysis of the literature, gallstone presence was only noted in 83.3% of patients where the presence of gallstones was assessed.

APBJ is also another risk factor of gallbladder malignancy[12]. Matsubayashi *et al*[12] reported a 72-year-old female patient with symptoms of abdominal pain. Laboratory investigations revealed raised alkaline phosphatase and gamma-glutamyl transpeptidase. CT scan confirmed a polypoid gallbladder mass. Magnetic resonance cholangiopancreatography scan showed ABPJ, and this was confirmed at subsequent endoscopic retrograde cholangiopancreatography. While APBJ is a well-known risk factor for gallbladder cancers[75], this was the first case of APBJ in GBCS noted in the literature.

Other risk factors mentioned include chronic cholecystitis, which could be both a risk factor and the manifestation of gallbladder malignancy. Unique to the gallbladder is a cycle of gallbladder epithelium damage and repair, enabling a chronic inflammatory environment from chronic cholecystitis[76]. This cycle of inflammation, injury, repair and regeneration increases cell turnover and oxidative stress. Yildiz *et al*[77] stated biliary tract to be the "consummate example of inflammation-associated carcinoma". Chronic inflammation from gallstone disease can lead to protein damage,

genetic mutations, inhibition of apoptosis, promotion of angiogenesis, modulation of cell adhesion and motility as well as immunosuppression. Chronic cholecystitis leads to gallbladder wall thickening, and CT or magnetic resonance imaging (MRI) scans are sensitive to detect wall thickness. However, it is not possible to distinguish if thickening of the gallbladder wall is due to inflammation or malignancy[78]. Thus, multidisciplinary discussion involving experienced radiologists and hepatobiliary surgical team is essential to make management plans for patients with suspicious gallbladder lesions.

Signs and symptoms

Clinical manifestations of GBCS are nonspecific, with symptoms such as abdominal pain localized to the right upper quadrant, constitutional symptoms, nausea, vomiting and fever. The mechanism resulting in constitutional symptoms in patients with cancer is multifactorial and not yet fully understood. It is thought that multiple pathways involving pro-cachectic and pro-inflammatory signals from tumor cells along with systemic inflammation of the host combine with widespread metabolic changes contribute to the manifestations of symptoms like anorexia and cachexia[79]. In particular, cholecystokinin is an integral peptide involved in satiety and regulating diet intake[80]. Given its role in gallbladder contraction, dysregulation of cholecystokinin could be involved in the manifestation of constitutional symptoms of anorexia in patients with GBCS.

Similarly, the pathophysiology of febrile response in malignancies is complex. Released pyrogenic cytokines from tumor cells and tissue macrophages induces a chain of events that result in reset of hypothalamic thermostat due to prostaglandin E2 and related pathways[81]. On physical examination, the presence of a right hypochondria tenderness or mass is not specific, and it does not rule out malignancy. Thus, if a patient is managed for suspected acute or chronic cholecystitis, a follow-up physical examination and imaging needs to be arranged to document resolution of inflammatory process. In this review, 2 asymptomatic patients were diagnosed with GBCS. From our analysis, Ishida *et al*[36] and Akatsu *et al*[46] reported incidental findings of GBCS on imaging findings for unrelated issues. Ishida *et al*[36] reported a 62-year-old female with unexpected calcification in the right upper abdomen in a CT meant for follow-up of percutaneous pinning of a left calcaneal fracture. Akatsu *et al*[46] reported a 76-year-old female who was on regular follow-up for cholelithiasis. Abdominal ultrasound revealed a heterogeneously hypoechoic mass around the gallbladder bed. In both patients, a preoperative diagnosis of possible gallbladder malignancy was made, and surgical exploration with subsequent cholecystectomy was performed.

Biochemical investigations

Biochemical abnormalities in GBCS are also mostly nonspecific. The most common derangements were transaminitis, hyperbilirubinemia and anemia. This was consistent with Ayoub *et al*[5] who reported that hepatic and inflammatory markers were often normal.

Presurgical diagnosis of gallbladder malignancies is difficult due to its varying presentations. Differentials to consider for such lesions when calcification is present include calcified gallstones, porcelain gallbladder and GBCS[78]. Our analysis noted cases where GBCS was initially diagnosed with cholelithiasis, acute cholecystitis, gallbladder empyema, diffuse peritonitis, pancreatic cancer and pyogenic liver abscess.

Imaging

As there are no radiological signs identified in the current literature that distinguishes GBCS from other gallbladder malignancies[50,51], the diagnosis is difficult even with imaging. For instance, Appelman *et al*[72] described a 91-year-old male presenting with yellow sclera, pale stools, dark urine and pruritus. His liver function tests were deranged with obstructive pattern, and a diagnosis of pancreatic cancer with biliary tract obstruction was made. The patient refused surgical intervention and died within 2 wk. Autopsy confirmed the diagnosis of metastatic disease with GBCS primary.

Khurram *et al*[4] reported a 64-year-old lady presented with right upper quadrant mass, intermittent fever and abdominal distension following a recent travel history to Ghana. CT scan revealed a hepatic lesion with coexisting gallbladder distension consistent with pyogenic liver abscess. Due to failure to respond to intravenous antibiotics, MRI scan was done. MRI scan showed a gallbladder fundus soft tissue lesion with local invasion into the liver[22]. Histopathological diagnosis of GBCS was

made after surgical excision. Hence, in the absence of a confirmatory preoperative diagnosis, all suspicious gallbladder lesions must be reviewed at multidisciplinary meetings.

Porcelain gallbladder, gallbladder tuberculosis and xanthogranulomatous cholecystitis are common benign conditions that can be confused with malignancy. Porcelain gallbladder is described as a hyperechoic focus with posterior acoustic shadowing on an ultrasound scan[82]. Ultrasound scan is not sensitive for regional and distant spread of malignancy. CT and MRI scans are more sensitive to detect contiguous spread to liver, regional lymph node involvement and distant metastases. Diffuse nodular thickening without layering, early enhancement, low apparent diffusion coefficient and high lesion to spinal cord ratio are MRI features suggestive of gallbladder cancer[83]. In addition, CT and MRI scans provide details that assist in surgical planning. 18-fluorodeoxyglucose-positron emission tomography-CT can aid in distinguishing between benign and malignant gallbladder lesions. Malignant lesions have high standardized uptake value. In a study reporting 30 patients with a mean age of 48.22 ± 31.33 years and gallbladder wall thickening (focal > 4 mm and diffuse > 7 mm), Gupta *et al*[84] reported that 18-fluorodeoxyglucose-positron emission tomography had high overall sensitivity (91%), specificity (79%), positive predictive value (77%), negative predictive value (92%) and diagnostic accuracy (84%).

Histological diagnosis

Diagnosis of GBCS is usually made after pathological analysis of a surgical specimen. In patients with unresectable neoplasms, tissue diagnosis can be achieved by percutaneous biopsy. This is essential to plan definitive chemotherapy[85]. In clearly resectable lesions, the role of percutaneous biopsy is debated due to risk of needle-tract seeding[86]. Furthermore, as the gallbladder is a hollow organ, bile spill and peritonitis remain a risk too[87]. As GBCS are rare tumors with poor prognostic outcomes, treatment options are not well defined, with little evidence supporting or refuting any postoperative adjuvant therapy. Okabayashi *et al*[88] and Mochizuki *et al*[8] both corroborate that surgical treatment remains the only cure for GBCS. While the histopathological features between GBCS and adenocarcinoma of the gallbladder are different, management is similar.

Surgical management

Currently, the consensus for treatment involves surgical excision of the gallbladder and extrahepatic bile duct, regional lymphadenectomy and even a pancreaticoduodenectomy depending on the extent of the growth[88]. Completion liver resection with or without lymphadenectomy and/or bile duct resection is an accepted standard for post simple cholecystectomy discovered gallbladder cancer with T_{1b} and higher stage. This approach not only involves two surgeries but also increases the risk of cutting through the tumor with potential for tumor seeding and dissemination. Yip *et al*[89] in a series of 40 patients with incidental gallbladder cancer reported that the majority of patients were not amenable for further curative resection. A report from Memorial Sloan-Kettering Cancer Centre involving 116 patients showed that survival of patients with residual disease was not different than survival of patients with stage IV disease, and neither group of patients benefit from reoperation[90]. Thus, single surgery may be better.

Radical cholecystectomy has higher morbidity as compared to simple cholecystectomy. Thus, the concept of something intermediate, *i.e.*, extended cholecystectomy, is attractive. Fujisaki *et al*[91] reported a case describing the concept of laparoscopic extended cholecystectomy with 1 cm liver margin; however, they proposed open conversion when intraoperative histology showed gallbladder cancer invading the subserosal layer. With current advancements, laparoscopic extended cholecystectomy was noted to have lesser intraoperative and postoperative complications than open extended cholecystectomy[92].

The key differences between a 'radical' and 'extended' cholecystectomy are restricting the liver parenchyma transection to the 2 cm wedge of liver tissue and performing regional lymphadenectomy and choledochectomy only in selected patients. Radical cholecystectomy can be done by open, laparoscopic or robot assisted approach, with comparable short-term outcomes[93]. Overall, more data is required to determine the safety and feasibility of minimal access techniques in gallbladder malignancies. Due to the absence of histological diagnosis, management of suspicious gallbladder lesions must be determined by local resources, surgeon experience and access to technology.

In a recent systematic review, Frountzas *et al*[94] reported that many patients with xanthogranulomatous cholecystitis were managed with complex procedures like wedge hepatic resection and bile duct excision with high open conversion rate (35.0%) at planned cholecystectomy. Intraoperative frozen section analysis is a useful adjunct in surgical planning. While intraoperative frozen tissue diagnosis is relatively reliable to determine whether lesions are benign or malignant, it does not reliably detail the depth of invasion of gallbladder malignancies[95]. Furthermore, the accuracy of intraoperative frozen tissue diagnosis for GBCS has yet to be determined due to paucity of scientific data.

Adjuvant treatment

The adjuvant treatment reduces recurrence risk and improves survival outcomes by eliminating or controlling the micrometastatic disease. A meta-analysis of retrospective studies including 6712 gallbladder cancer patients reported that lymph node positive patients enjoyed the survival benefit[96]. Few reported patients consider the use of UFT: tegafur/uracil, gemcitabine or a combination of tegafur/gimeracil/oteracil. The median survival of GBCS is 7.8 mo[10], and the addition of such regimes has not shown to improve survival[38]. There is a report by Pu *et al*[38] of using a combination of 5-FU (commonly used in gallbladder cancer) and oxaliplatin (commonly used in sarcomas). They reported a 59-year-old female coming in with right upper quadrant pain, fever and a raised CA19-9 level of 12000 U/mL, which was confirmed to be GBCS. The patient received oxaliplatin 150 mg and 5-FU 500 mg intravenously every 30 d for 6 cycles. At 6-mo follow-up, she did not reveal any signs of recurrence.

Adjuvant radiotherapy is shown to be of value in reducing local recurrence in selected patients with gallbladder cancer. In a study including 4180 patients with resected gallbladder cancer diagnosed from 1988 to 2003 from the Surveillance, Epidemiology, and End results database, Wang *et al*[97] reported that adjuvant radiotherapy provides survival benefit in node positive or T2 and higher stage disease. A single arm phase II study conducted by South West Oncology Group reported that gemcitabine plus capecitabine, followed by radiation (45 Gy to regional lymphatics, 54-59.4 Gy to tumor bed) and capecitabine resulted in 56% 2-year survival rate for patients with gallbladder cancer. Based on these results, the American Society of Clinical Oncology guidelines recommend chemotherapy plus radiation in gallbladder cancer patients with R1 resection[98]. There is no data to support neoadjuvant chemotherapy. Due to aggressive biological behavior, rapid progression or recurrence is common, and this is associated with a myriad of constitutional symptoms. For holistic care, management of the patients' subjective symptoms of anorexia and lethargy needs to be considered. Testosterone replacement therapy helps alleviate such symptoms in male patients with advanced cancer[99].

Prognosis

Generally, the prognosis of GBCS is poor. The majority of patients presenting to the hospital are locally advanced, with liver metastasis and peritoneal dissemination. Other metastasis sites reported include adrenal glands, pancreas, diaphragm and the lower thoracic vertebrae. Zhang *et al*[3] reported a mean survival time of 17.5 mo, with 1-year and 5-year survival rates at (19 ± 5)% and (16 ± 5)%, respectively. While it was previously noted the longest survival time to be reported as 54 mo by Uzun *et al*[44], our review noted 86 mo to be the longest survival time[10]. Aldossary *et al*[10] reported a 62-year-old female patient who complained of severe intermittent right upper quadrant pain of 2 mo duration. Laboratory investigations were normal, and ultrasound suggested a gallbladder with large stones and a non-mobile echogenic mass. A stage II (pT2, pN0, M0) moderately differentiated GBCS was noted on histology after laparoscopic cholecystectomy. The patient underwent 14 cycles of adjuvant chemotherapy. She had local recurrence at 2 years. Wide local excision of the mass with wedge resection of the liver, lymphadenectomy and partial gastrectomy was done. The patient remained disease free for 86 mo. Zhang *et al*[3] also claim that tumors smaller than 5 cm had a more prolonged survival, however we did not observe this. More data is required to confirm this, as only 28 patients detailing both the survival data and size of tumor have been reported.

Role of tumor markers

GBCS is not noted to have association with any tumor markers. Consistent with the current literature, most of the patients did not note any raised tumor markers[5]. However, it is still common practice for physicians to perform tumor marker levels

such as CA19-9, carcinoembryonic antigen and alpha-fetoprotein when considering possible differentials for masses in the gallbladder as well as for prognostication. For instance, Hayashi *et al*[100] propose that alpha-fetoprotein-producing carcinomas of the gallbladder are more likely to metastasize to the liver and have poor prognosis. CA19-9 is typically associated with pancreatobiliary malignancies but has a limited role in clinical practice[101]. Thus, prognostication is relied typically on histological features, pathologic stage as well as immunohistochemistry. Immunohistochemistry for the mesenchymal and epithelial components yield positive staining for vimentin and cytokeratin[45]. Our review shows that the majority of the patients had positive staining for vimentin (81.2%) and cytokeratin (79.2%). Additionally, Ki-67 was suggested by Kubota *et al*[45] to have prognostic value, whereby its presence signifies a possibly higher malignant proliferative potential for GBCS. However, this claim needs to be further investigated as Kubota *et al*[45] examined this immunohistochemical marker in only 1 patient with CSGB.

Comparison to gallbladder adenocarcinoma

There is substantial overlap of risk factors, diagnosis and treatment of GBCS with gallbladder adenocarcinoma. Thus, the majority of authors extrapolate the clinical characteristics of gallbladder adenocarcinoma to determine the best approach to diagnosis and management of GBCS. From this review, we can determine three key differences between GBCS and gallbladder adenocarcinoma. First, tumor markers have limited utility in patients with GBCS. In a study of 55 cases by Shukla *et al*[102], it is noted that the combination of CA-125 and CA19-9 helped detect gallbladder malignancy in patients with gallstones (80.7%). Second, the prognosis of GBCS may be marginally better compared to carcinoma of the gallbladder. In the meta-analysis by Zhang *et al*[3], it was noted that the survival rate was slightly better (16% ± 5% 5-year survival) compared to carcinoma of the gallbladder (0-10% 5-year survival). Thus, the identification of GBCS will be useful to determine the prognosis for patients albeit with only a small variation between the two. Third, immunohistochemistry markers like vimentin and cytokeratin are associated with diagnosis of GBCS.

CONCLUSION

In conclusion, GBCS is more common in females. Gallstones and chronic cholecystitis are risk factors for GBCS. Serum biochemistry and tumor markers have a limited role in diagnosis. Typical imaging modalities can assist to establish a diagnosis in patients with suspicious gallbladder lesions. Multiple imaging modalities are complementary. Multidisciplinary oncology board discussions are essential to guide management plans. Surgery is currently the only curative option for GBCS, and size of the tumor does not impact prognosis. While most features of GBCS parallel that of carcinomas of the gallbladder clinically, identification of GBCS specifically allows clinicians to determine overall prognosis. Due to paucity of reported cases, more evidence is required before meaningful and valid evidence-based patient-centric recommendations can be made. This review serves to educate and raise awareness among the clinicians dealing with gallbladder malignancies. It is likely that there are more clinical differences between GBCS and common forms of gallbladder cancer; active reporting of cases will help enhance understanding of this rare cancer.

ARTICLE HIGHLIGHTS

Research background

Literature on gallbladder carcinosarcoma (GBCS) is currently scarce, with less than 100 cases reported since the first case by Karl Lansteiner.

Research motivation

While there has been efforts by Zhang *et al* in 2008 to consolidate the literature, there has not been a review of the current literature since.

Research objectives

This study aims to fill this gap by providing a comprehensive overview of GBCS.

Research methods

A literature review was performed on the PubMed database using the keywords "Gallbladder" AND "Carcinosarcoma" from 1970 to 2021, where relevant articles were included. Animal studies, gallbladder carcinoma and non-gallbladder pathology as well as articles that were not in English or Japanese were excluded.

Research results

GBCS is more common in females. Gallstones and chronic cholecystitis are risk factors for GBCS. Serum biochemistry and tumor markers a have limited role in diagnosis. Typical imaging modalities can assist to establish a diagnosis in patients with suspicious gallbladder lesions. Multiple imaging modalities are complementary. Multidisciplinary oncology board discussions are essential to guide management plans. Surgery is currently the only curative option for GBCS, and size of the tumor does not impact prognosis.

Research conclusions

While most features of GBCS parallel that of carcinomas of the gallbladder clinically, identification of GBCS specifically allows clinicians to determine overall prognosis. Due to paucity of reported cases, more evidence is required before meaningful and valid evidence-based patient-centric recommendations can be made.

Research perspectives

Due to the paucity of the number of reported cases, more active reporting of such should be encouraged to further understand this malignancy.

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