ISSN 2218-4333 (online)

World Journal of *Clinical Oncology*

World J Clin Oncol 2022 August 24; 13(8): 663-728

Published by Baishideng Publishing Group Inc

World Journal of Clinical Oncology

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World Journal of Clinical Oncology

Monthly Volume 13 Number 8 August 24, 2022

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NAME OF JOURNAL
World Journal of Clinical Oncology

ISSN
ISSN 2218-4333 (online)

LAUNCH DATE
November 10, 2010

FREQUENCY
Monthly

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PUBLICATION DATE
August 24, 2022

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World Journal of Clinical Oncology

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World J Clin Oncol 2022 August 24; 13(8): 663-674

DOI: 10.5306/wjco.v13.i8.663

ISSN 2218-4333 (online)

FIELD OF VISION

Potential of mRNA vaccines to become versatile cancer vaccines

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Specialty type: Oncology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Jiraviriyakul A, Thailand; Korbelik M, Canada

Received: March 29, 2022
Peer-review started: March 29, 2022
First decision: May 31, 2022
Revised: June 15, 2022
Accepted: July 25, 2022
Article in press: July 25, 2022
Published online: August 24, 2022

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Abstract

For centuries, therapeutic cancer vaccines have been developed and tried clinically. Way back in the late 19th century, the Father of Immunotherapy, William Coley had discovered that bacterial toxins were effective for inoperable sarcomas. In the 1970s, the Bacillus Calmette-Guérin (BCG) vaccine was repurposed, *e.g.*, for advanced melanomas. Then, therapeutic cancer vaccines based on tumor-associated antigens (found on the surfaces of cancer cells) were tried clinically but apparently have not made a really significant clinical impact. For repurposed pathogen vaccines, only the BCG vaccine was approved in 1989 for local application to treat nonmuscle-invading bladder cancers. Although the mildly toxic vaccine adjuvants deliberately added to conventional pathogen vaccines are appropriate for seasonal applications, when repurposed for continual oncology usage, toxicity may be problematic. In 2010, even with the approval of sipuleucel-T as the very first cancer vaccine (dendritic cell) developed for designated prostate cancers, it has also not made a really significant clinical impact. Perhaps more "user friendly" cancer vaccines should be explored. As from approximately 30 years ago, the safety and effectiveness of mRNA vaccination for oncology had already been studied, the current coronavirus disease 2019 pandemic, though disastrous, has given such progressively advancing technology a kickstart. For oncology, other virtues of mRNA vaccines seem advantageous, *e.g.*, rapid and versatile development, convenient modular design, and entirely cell-free synthesis, are being progressively recognized. Moreover, mRNAs encoding various oncology antigens for vaccination may also be tested with the combination of relatively non-toxic modalities of oncology treatments, *e.g.*, metformin or metronomic (low-dose, prolonged administration) chemotherapy. Admittedly, robust clinical data obtained through good quality clinical trials are mandatory.

Key Words: Cancer vaccine; Cyclophosphamide; Metformin; Metronomic chemotherapy; mRNA vaccine; Myocarditis; Tumor microenvironment

Core Tip: Although vaccines are effective for pathogen prevention and cancers, hitherto, oncology vaccines have not yet made a very significant clinical impact. Currently, as mRNA vaccines already have a proven safety profile, it is highly appropriate to further develop the decades-old mRNA technology for oncology. Compared to other approved cancer vaccines, oncology mRNA vaccines may be more versatile, pragmatic, affordable, and effective. To combat the notoriously resistant tumor microenvironment, the probable mutual enhancement effects with, *e.g.*, metronomic chemotherapy should be fully explored, especially as no significant added toxicity is anticipated. Clearly, undertaking much more research work (especially clinical) is mandatory.

Citation: Tsao SY. Potential of mRNA vaccines to become versatile cancer vaccines. *World J Clin Oncol* 2022; 13(8): 663-674
URL: https://www.wjgnet.com/2218-4333/full/v13/i8/663.htm
DOI: https://dx.doi.org/10.5306/wjco.v13.i8.663

INTRODUCTION

The term "cancer vaccine" includes vaccines, pathogen, or otherwise that induces the innate and adaptive immunities for specific purposes; as such, it does not include items like oncolytic viruses. Although cancer immunotherapy is now well recognized to be a significant modality of treatment, interestingly, way back in the 1890s, an American orthopedic surgeon, William Coley had already documented an unexpected regression of a sarcoma when a surgical wound failed to close due to wound infections. Coley hypothesized that the tumor regression may be related to the patient's febrile erysipelas infection (caused by *Streptococcus pyogenes* bacteria). Eventually, he had developed the very first cancer vaccine containing toxins from killed *Streptococcus pyogenes* and *Serratia marcescens* bacteria [1]. However, with variable successes across other patients, Coley's approach unfortunately waned in popularity especially upon the advent of radiotherapy and the then very novel cancer chemotherapy. Nevertheless, he had most remarkably pioneered the concept of bacterial toxins inducing immunity that was also effective for eradicating cancer cells. For this, he was subsequently honored as the "Father of Immunotherapy". Rather unfortunately, cancer vaccines had not subsequently made very significant clinical impacts since then. Actually, specific cellular vaccines have been made to induce satisfactory immune responses against cancer cells, for instance, autologous cell-based cancer vaccines, *e.g.*, for hematological and other cancers[2,3]. However, these may be less versatile, too time consuming to process, and too costly to exert a significant impact on a good number of cancer patients. Apparently, as the very first cancer vaccine [dendritic cell (DC)] developed for specific prostate cancers (sipuleucel-T) has also not made a really significant clinical impact, it may be appropriate to explore other more "user friendly" cancer vaccines.

TUMOR-ASSOCIATED ANTIGENS

Remarkably, cancer immunotherapy in the form of cancer therapy vaccines (that followed William Coley's discoveries) waxed and waned probably because both radiotherapy and chemotherapy were developing steadily by then. Yet, by the 1970s, the Bacillus Calmette-Guérin (BCG) vaccine had actually been repurposed for cancer therapy and tried clinically, *e.g.*, for melanoma[4]. Another form of immunotherapy involved stimulating the cancer patient's own innate and adaptive immunities using the cancer's tumor-associated antigens (TAAs) for developing cancer vaccines. Basically, TAAs are related to antigen molecules present on tumor cell surfaces, *e.g.*, embryonic proteins and glycoprotein antigens. These have been exploited to develop TAA cancer vaccines[4]. However, even though most TAAs are being overexpressed on cancer cells, they are actually not specific enough as these antigens are also expressed in normal tissues[5]. Thus, as TAAs may arise, *e.g.*, as oncofetal antigens, a peripheral tolerance may have already developed to these antigens and would thus preclude a satisfactory immune response to TAAs. Admittedly, despite the encouraging evaluation of numerous vaccine strategies targeting various tumors, the efficacy of therapeutic cancer vaccines has not been clearly demonstrated through robust clinical trials[6]. Notably, most of the tumor antigens employed for cancer vaccines were non-mutated, overexpressed self-antigens, eliciting mostly T cells having low-affinity T cell receptors (TCRs) that were deemed the most appropriate to mediate an effective anti-tumor response. Taken together, TAA cancer vaccines have not yet made a significant clinical impact on cancer control[6,7].

REPURPOSING PATHOGEN VACCINES

Repurposing pathogen vaccines for oncology has also been proposed as a feasible modality of cancer immunotherapy. Actually, even in the 1970s, the BCG vaccine had already been tried clinically, *e.g.*, for melanoma[4]. It was felt that, despite some demonstrable effect due to BCG, it did not seem to influence significantly the course of the advanced melanoma. Subsequently, pre-clinical studies of some other pathogen vaccines seemed to be more encouraging. However, it was found that possibly, one of the best applications was intratumoral administrations of pathogen vaccines to turn "cold" tumors into "hot" ones, *i.e.*, having more abundant immune cells (see Section "Tumor Microenvironment"). Admittedly, although this may be very helpful, low-dose cyclophosphamide injected more conveniently through tie intravenous route, would also have a similar effect[8]. Currently, of all the pathogen vaccines, only BCG is approved in 1989 for local treatment of nonmuscle-invasive bladder cancers, even though the exact mechanism is still controversial[9,10].

Importantly, although the mildly toxic vaccine adjuvants deliberately added to conventional pathogen vaccines to boost the immune response was appropriate for seasonal application, continual oncology usage may be controversial[11]. Even though aluminum salts are the commonest vaccine adjuvants, in extreme cases, heavy metal poisoning may occur especially if very frequent administrations of these repurposed pathogen vaccines are given. Currently, the potential toxicity of aluminum is increasingly recognized[12]. Perhaps intratumoral administrations would be most appropriate, except for the fact that the mode of administration is technically more complicated[13,14]. Taken together, with repurposing of pathogen vaccines for oncology, the frequency of administration should be noted well. For instance, a study administering a weekly combination of several repurposed pathogen vaccines for lung cancer [NCT02333474] might have problems related to vaccine adjuvant toxicity.

MRNA VACCINES

Remarkably, during this coronavirus disease 2019 (COVID-19) pandemic, mRNA vaccinations have demonstrated their remarkable success and good safety profile. mRNA for incorporation into vaccines is synthesized *in vitro* to mimic the host mRNA in order to increase mRNA stability and translation efficiency[15]. Moreover, unlike conventional pathogen vaccines, mRNA vaccines are devoid of any cellular or animal components. Additionally, some mRNA vaccines do not require any adjuvants to boost their immune effectiveness[16]. As booster pathogen mRNA vaccines are often given at intervals of 5 mo or less for healthy subjects, when applied for cancer patients, repeated applications would most probably be feasible even at shorter intervals. This is especially so for those mRNA vaccines that have no added adjuvants. Of course, more robust data upon further clinical studies are required for confirmation.

DEVELOPMENT OF MRNA VACCINES

Currently, three major types of mRNA vaccines are available: (1) Conventional, non-amplifying mRNA molecules; (2) Base-modified, non-amplifying mRNA molecules incorporating chemically modified nucleotides; and (3) Self-amplifying mRNAs (saRNAs) that maintain the auto-replicative activity derived from an RNA virus vector. Thus, saRNAs would encode both the antigen and the viral replication machinery which enables intracellular RNA amplification and ample protein expression[17]. saRNAs may thus be advantageous as they maintain all the advantages of mRNA vaccines (rapid development, convenient modular design, and entirely cell-free synthesis), let alone a significantly lower dose of mRNA is now feasible, due to the self-replicating properties[17].

Admittedly, despite much work on TAA vaccines, there are still no very significant clinical impacts. On the other hand, mRNA vaccines may generate potent and protective immune responses of both cellular and humoral types. Basically, mRNA is an intermediate between the translation of protein-encoding DNA and protein production[16]. Notably, unlike pathogen vaccines, adjuvants to enhance vaccine immunity are no longer essential and so, repeated administration for oncology therapy would unlikely be problematic. Moreover, through billions of administrations of mRNA vaccines, the safety profile can be better confirmed. Lastly, it is also most unlikely to have any chance of incorporation into potential oncogenic sites within the genome[15].

REFINING MRNA VACCINES

Although pioneer mRNA vaccines (for oncology) were naked, *e.g.*, the version employed by a German group, subsequent work had appropriately enabled encapsulation in a lipid nanoparticle (LNP)[18,19]. This effectively limits detection by the innate immune system, enhances the cellular uptake of the

mRNA, and prolongs as well as enhances protein expressions. Moreover, the ionizable cationic lipid can also improve the release of mRNA from the endosome to the cytoplasm and markedly prolong protein expressions[16]. Importantly, encapsulation may also serve as a self-adjuvant purpose (see below).

For administration, they can be injected subcutaneously, intradermally, or directly into lymph nodes or tumors. Notably, the production of mRNA vaccines is potentially faster, more flexible, and less expensive and can even be used for precise and individualized therapies. During this pandemic, the rapid and safe vaccine production was clearly shown[16]. Vaccine adjuvants are usually not required as the LNP already induces an innate immune response – a self-adjuvant. With continual mRNA vaccine development, the structured 5′ as well as 3′ termini and the double-stranded RNA replication intermediates of saRNA vaccines would be recognized, leading to a type I interferon (IFN1) response (see below). Remarkably, this immune stimulation would serve as a self-adjuvant to increase vaccine immunogenicity[15].

Lastly, vaccine quality may be improved by nucleoside modification or complexed mRNAs, and further shaped or influenced by the choice of the delivery routes and formats, *e.g.*, LNP vaccines. It was also found that the introduction of noninflammatory modified nucleosides into the mRNA was advantageous as they induce potent T follicular helper and also germinal center B cell responses[20].

TWO MRNA VACCINES FOR COVID-19

By December 2020, two mRNA vaccines from BioNTech/Pfizer and Moderna were approved against the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus, representing the very first approval for any mRNA vaccines. For development, BioNTech/Pfizer had previously compared several RNA-based COVID-19 pandemic vaccine candidates in clinical studies in Germany and the US. Despite incomplete data publication of all technical details then, mRNA vaccines were known to be LNP-formulated and nucleoside-modified. Eventually, two most promising vaccine candidates were selected: BNT162b1 (encoding the SARS-CoV-2 receptor–binding domain and BNT162b2 (encoding a modified version of the SARS-CoV-2 full-length spike protein)[21]. As BNT162b2 was found to exhibit a good balance of efficacy and safety even at a low dose of 30 μg, it attained the international phase 2–3 clinical trials[22]. Approximately 44000 adults were subjected to two intramuscular injections of 30 μg of BNT162b2 (21 d apart) (NCT04368728). That regimen could confer 95% protection against SARS-CoV-2. Moreover, the titers of SARS-CoV-2–neutralizing antibodies either resembled or exceeded those found in patients who had recovered from COVID-19[16].

SAFETY ASPECTS OF MRNA FOR COVID-19

Before 2020, no mRNA vaccine had ever been approved. During this pandemic, with the authorization and approval of mRNA vaccines against SARS-CoV-2, the safety profile become better recognized. As mRNA vaccines are devoid of any cellular or animal components, they have since been shown to be generally safe and well-tolerated. Integration into the subjects' genome is deemed not possible. With storage at very low temperatures, microbial contaminations are also extremely unlikely. Serious adverse effects were few, although local pain and redness at the injection sites may occur. Rarely, systemic allergic reactions may also ensue. Besides systemic inflammatory reactions, a theoretical risk of inflammation and autoimmunity may occur, likely due to the induced IFN1 response[23]. In patients with systemic lupus erythematosus or other similar autoimmune diseases, anti-self RNA antibodies may develop and could worsen their autoimmunity[24]. Moreover, toxic side effects related to the delivery compounds or complexing agents and potentially, to inserted nucleotides may occur[16]. Although rare, a serious adverse event of myocarditis and/or pericarditis may occur especially in younger adults and adolescents, predominantly in males (12.6 cases/million doses of the second-dose mRNA vaccine) – a few days after the dose, chest pain may occur, with ECG changes, raised serum cardiac troponin levels, and myocarditis features on MRI. Although the mechanism is unclear, it mostly resolves spontaneously [25]. Obviously, many more long-term clinical studies on a wider population spectrum are mandatory.

EFFECT OF EXOGENEOUS MRNA ON IMMUNITY

Upon confirmation that exogenous mRNA is being processed as for any endogenous mRNA, for more efficient T cell activation, a costimulatory signal was found to be helpful for inducing a better immune response with consecutive cytokine production[26]. Naturally, DCs would express these costimulatory signals (such as B7 molecules) after sensing pathogen-associated molecular patterns (PAMPs) that indicate microbial infection or imminent danger. Pharmacologically, this can be achieved by exploiting toll-like receptor (TLR) ligands[27]. TLRs are related to the innate immune system's ability to detect PAMPs. Induction of IFN1 by viruses or other pathogens is indispensable for innate immune responses

and would thus confer anti-microbial activities[28]. Upon sensing PAMPs, an immediate innate inflammatory response (including IFN1 induction) is initiated. Exogeneous mRNAs are likewise sensed by TLRs and double-strand RNAs can induce a strong IFN1 response. Eventually, clonal expansion of antigen-specific B and T cells results in target cell elimination. Although this may be less complicated for infection prophylaxis, significant problems abound for effective control of advanced cancers. Conceptually, resistant cancers often have immunosuppressive tumor microenvironments (TMEs) through recruiting myeloid-derived suppressor cells (MDSCs), regulatory T cells, macrophages, *etc*., to the TME, let alone the production of immunosuppressive cytokines. Hence, very much more robust modalities of treatments than infection prophylaxis are called for (see Section "Tumor Microenvironment").

PIONEERING MRNA TECHNOLOGY FOR ONCOLOGY

Remarkably, even as early as 1995, the feasibility of using mRNA technology for oncology was noted [29], with mRNA transcripts encoding luciferase and human carcinoembryonic antigen (CEA) then. Subsequently, DCs were transfected with either mRNA encoding TAAs[4] or mRNA technology was employed for *in vivo* induction of T cell immunity[30]. Notably, DCs could utilize mRNA encoded TAAs for the induction of anti-cancer immunity[31]. Throughout the decades, more and more research work had validated the feasibility and possible efficacy of mRNA vaccination for immunotherapy against cancers.

Eventually, knowledge on mechanisms involved in innate and adaptive immune sensing ensued. Moreover, various novel approaches of mRNA delivery and complexing of the vaccine could be implemented. Undoubtedly, these may have paved the way to current successful clinical trials on COVID-19. Even for oncology mRNA vaccines, these may be generated using *ex vivo* loaded or electroporated DCs, usually with a known carrier. DCs are then isolated and subsequently transfected with mRNA encoding, *e.g*., TAA(s) before re-infusion into the patient. For instance, transfection by electroporation has been found to be safe for cancer patients[32]. DCs electroporated with mRNA encoding ovalbumin or tumor-derived mRNAs can actually generate robust tumor-specific immune responses in different murine melanoma models and even in patients undergoing vaccination trials (see Table 1).

EARLY CLINICAL TRIALS OF MRNA FOR ONCOLOGY

In 2002, oncology mRNA vaccines for enhancing immunity were reported to be useful for a patient with a carcinoembryonic antigen-expressing adenocarcinoma[33]. As mRNA was known to be basically a copy of the coding genomic information, it was thus found to be useful for the expression of therapeutic proteins[18]. In mice, naked mRNA coding for tumor antigens was administered by injecting intradermally. Most interestingly, it resulted in protein expression as an immune response. Subsequently, the same protocol was applied to 15 melanoma patients in the first phase I/II trial and found to be safe[19]. Notably, some patients even had an increase in antitumor humoral immune response. After the injection of the mRNA cancer vaccine, the encoded protein was translated and presented to the immune system – closely resembling the natural course of a viral infection and its consecutive induction of a protective immune response. Importantly, upon entering to the cytoplasm, the exogenous mRNA had been found to be processed as for any endogenous mRNA[16].

THERAPEUTIC MRNA VACCINES: INJECTION SITES

Although pathogen mRNA vaccines are nearly always given intramuscularly, there are various other different routes for therapeutic mRNA vaccines. These other injection sites may impact on the induced immune response. As the human skin has many antigen-presenting cells (APCs), especially interstitial DCs in the dermis[34], after intradermal injection, exogenous mRNAs are taken up and locally expressed by ample APCs there. Despite scanty immune cells in muscles, circulating immune cells would eventually reach the injection site to process and present the antigen locally. This is just like the expected actions caused by traditional pathogen vaccine adjuvants. It is where the usual local inflammatory reaction at injection sites promotes significant immune cell activities[16]. Even as technical details are beyond the scope of this article, upon local injection of mRNA vaccines, mRNAs will eventually be processed by APCs reaching there and antigen-specific CD8+ T cells are induced.

IMMUNOGENIC CELL DEATH

It is increasingly recognized that the cancer cell killing by chemotherapy (ChT) is not just by direct

Table 1 Selected national registered clinical trials on combination mRNA oncology vaccines[a]

mRNA vaccine	I.S.	Combo agent	Ph	Cancer	Oncol status	Yr	Country	Trial status	NCT number
mRNA-2752	i.t.	+ durva	1	Solid ca, lymph	R/R	2018	United States	Recruiging	03739931
BI 1361849	i.d.	+ durva +/- treme	1/2	NSCLC	Adv	2017	United States	Completed	03164772
mRNA-4157	i.m.	+/- pembro	1	Solid ca	Resected	2017	United States	Recruiting	3313778
mRNA-5671/V941	i.m.	+/- pembro	1	NSCLC/ CRC/ pancCA	Adv	2019	United States	Not yet recruiting	03948763
TriMix[b]	i.t.	Neoadj ChT +/- TriMix[b]	1	Breast	Early	2018	Belgium	Recruiting	03788083
W_ova1	i.v.	+ neoad + adj ChT	1	Ovarian ca	Early	2019	Nether- lands	Recruiting	04163094
W_pro1	i.v.	+/- cemip	1/2	mCRPC	Adv	2020	United States	Recruiting	04382898
Trivalent DCs[c]	i.d.	TMZ/RT +/- DCs	2/3	GBM	Post-op	2018	Norway	Recruiting	03548571
PSCT19[d]	i.v.	allo-SCT +/- PSCT19[d]	1/2	Hemat	Post- allo-SCT	2015	Nether- lands	Completed	02528682
WT1 DC	i.d.	adj TMZ +/- WT1 DC	1/2	GBM	Post-op	2016	Belgium	Recruiting	02649582
pp65 DC[e]	i.d.	adj TMZ +/- pp65 DC[e]	2	GBM	Post-op	2015	United States	Recruiting	02465268
pp65 DC[f]	i.d.	+/- varli	2	GBM	Post-op	2018	United States	Recruiting	03688178
RO7198457	i.v.	+/- pembro	2	Melanoma	Adv	2019	United States	Not yet recruiting	03815058
RO7198457	i.v.	+/- atezo	1	Solid tumors	Adv	2017	United States	Not yet recruiting	03289962

[a]For combinations with therapeutic mRNA vaccines, in principle, the best candidates are those without immune suppressive properties, *e.g.*, while maximum tolerated dose chemotherapy (ChT) may suppress immunity induced by mRNA vaccines, ironically, mChT could have the opposite effect of priming resistant tumors to be more responsive ones[8,63].
[b]CD40L, CD70, and constitutively active toll-like receptor 4.
[c]Dendritic cells (DCs) transfected with mRNA of neoantigens (survivin, hTERT) and autologous tumor stem cells.
[d]PSCT19: MiHA-loaded PD-L-silenced DC Vaccination.
[e]pp65-shLAMP mRNA (autologous) DCs with GM-CSF.
[f]Human CMV pp65-LAMP mRNA-pulsed autologous DCs. Adj: Adjuvant; Adv: Advanced; Allo-SCT: Allogeneic stem cell transplantation; Atezo: Atezolizumab; Ca: Cancer; Cemip: Cemiplimab; ChT: Chemotherapy; CRC: Colo-rectal cancer; Durva: Durvalumab; GBM: Glioblastoma multiforme; Hemat: Hematological malignancies; i.d.: Intradermal; I.S.: Injection site; i.t.: Intratumoral; Lympho: Lymphoma; mCRPC: Metastatic castration-resistant prostate cancer; MTD: Maximum tolerated dose; Neoadj: Neoadjuvant; NSCLC: Non-small-cell lung cancer; Oncol: Oncology; PancCA: Pancreatic cancer; Pembro: Pembrolizumab; Ph: Phase; Post-op: Post-operative; R/R: Relapsed/residual; RT: Radiotherapy; SCT: Stem cell transplant; TMZ: Temozolomide; Treme: Tremelimumab; Varli: Varlilumab.

cytotoxicity, but also by restoring immunity primed by the mechanism of immunogenic cell death (ICD). Intriguingly, dying cancer cells may be immunogenic provided that they emit a set of immunostimulatory signals inducing an activation of intracellular stress response pathways. As the phenomenon of ICD has already been described elsewhere, it is not repeated in this perspective article[35,36]. Briefly, ICD is characterized by cancer cell killing through cell-surface translocation of calreticulin (CRT), extracellular release of ATP and high mobility group box 1 (HMGB1), as well as stimulation of IFN1 responses. For ICD, emission of signals or "damage-associated molecular patterns" (DAMPs) is required. It is akin to a significant quantity of specific cancer cell death debris that may induce strong immune effects. Although ICD is a very attractive oncology phenomenon, maximum tolerated dose chemotherapy (MTD ChT) may have suppressed much of the immunity so induced, and metronomic chemotherapy (mChT) is preferred[37,38]. Moreover, certain mChT agents, *e.g.*, cyclophosphamide, also induce ICD itself (see Section "Combining mRNAs with Metronomic Chemotherapy"). Notably, the tumor infiltrating lymphocytes (TILs) are also modulated and would reactivate antitumor immunity within the notorious and immuno-suppressive TME.

TUMOR MICROENVIRONMENT

Most advanced cancers would deliberately produce a TME to disable and evade the body's immunity. The TME is now well recognized to be the main culprit for the vast majority of cancer resistance. With a serious lack of essential nutrients, *e.g.*, glucose, and oxygen, infiltrating immune cells are thus starved in this deliberately hostile environment[39]. Yet, cancer cells in the TME manage to survive readily through consuming minimal nutrients. Moreover, they also can manage by-products to their own advantage, *e.g.*, lactic acid which can reduce immune cell functions to the cancer cells' advantage. Taken together, the TME is most elusive and resilient and has various "plan Bs" and "plan Cs" to enable an almost intractable resistance to most conventional oncology treatments, especially immunotherapy, except perhaps, some immune checkpoint inhibitors (ICIs, see below). To tackle such TMEs, a multi-prong approach is most appropriate.

Notably, tumors having a robust TME may also be described as "cold" tumors, being unresponsive to most oncology treatments, whereas "hot" tumors are the exact opposite. Now, various innovative methods may be required to render such "cold" tumors into "hot" ones (having abundant immune cells). Notably, intratumoral mRNA vaccines might turn "cold" tumors into "hot" ones[40]; similarly, low-dose intravenous cyclophosphamide has also been found to act likewise[8]. This mechanism may be very useful for resistant tumors as there may be a much desired effect of "mutual enhancement" to tackle tumors which would go hand-in-hand with very evasive TMEs.

COMBINING MRNA VACCINES WITH IMMUNE CHECKPOINT INHIBITORS

In this era of cancer immunotherapy, ICIs have been widely applied for managing cancer patients. Although ICIs do not share similar toxicities with cancer ChTs, ICIs have their own disadvantages as has been discussed elsewhere[36]. Briefly, the response rates are too low and the adverse effects (mostly autoimmune related) may also be significant, so much so that patients with pre-existing autoimmune disorders are deprived of the benefits of ICIs. Realistically, the majority of cancer patients would not derive any benefit from ICIs. Moreover, the "one size fits all" dosage commonly approved for ICI prescriptions may be associated with higher adverse effect rates, especially in Asians who usually have smaller body builds than Caucasians. Although the combination with mRNA cancer vaccines might be beneficial [NCT03948763 (see Table 1)], *e.g.*, to raise the response rates, as both modalities of treatments are immune related, whether immune-related adverse effects might be even more common would require careful documentation, even as the higher cost of such combinations could be ignored.

COMBINING MRNAS WITH METRONOMIC CHEMOTHERAPY

Recently, the advantages of using mChT as one of the ways to patch up immunotherapy deficits have been detailed elsewhere[36]. Briefly, mChT agents not only act akin to targeted therapy agents but are also much less toxic than MTD ChTs so that they would not suppress immunity generated by combination cancer immunotherapy agents. Actually, as some ChT agents have the ICD phenomenon, immunity is enhanced (see Section "Immunogenic Cell Death"). Although MTD ChT has been designed to achieve maximum cancer cell killing, such very high dosages would likely suppress any immunity so generated, be it by mRNA vaccines or by the ICD phenomenon. Thus, as mChT usually does not suppress immunity, it is more appropriate for these combinatory purposes.

Intriguingly, mChT, *e.g.*, very short courses of intravenous low-dose cyclophosphamide, may ironically have a useful action of turning "cold" tumors "hot"[8]. For cyclophosphamide, the personal experience[36] and others[42] tally with such an action, even though the mechanism was entirely unknown decades ago. Importantly, the current evidence is on enhancing immunity mainly by modifying regulatory T cells (Tregs). mChT may even prime "cold" tumors into "hot" ones (see Section "Tumor Microenvironment"). Coincidentally, mRNA vaccines can also act likewise[40] so that there would now be a most desirable mutual enhancement effect. Such combinations are highly worth exploring further, especially as currently, mRNA vaccines may become a potential oncology breakthrough – thus, mChTs with ICD mechanisms[36] would work hand-in-hand with mRNA vaccines for the desired mutual enhancement effect. Although far too few clinical trials have been done on its combination, the remarkable safety profile of mChT is advantageous as no untoward toxicities are expected upon the combination.

COMBINING MRNA VACCINES WITH METFORMIN

Another similar agent deemed suitable for combination with mRNA vaccines is metformin. It has a similarly good safety profile as mChTs[43]. The details have already been reviewed elsewhere[41].

Briefly, despite its discovery around 100 years ago as an anti-diabetic, it is recently known as an agonist of the adenosine monophosphate-activated protein kinase (AMPK) that inhibits the mammalian target of rapamycin (mTOR), especially as mTOR is activated in cancer cells and would even convey drug resistance[44]. Metformin also has an ability of preferentially targeting cells that have abnormal or altered glycolysis, including cancer associated fibroblasts (CAFs). These cells may thus be rendered more susceptible than other cells to the action of cisplatin ChT[45]. This is valuable as CAFs play a vital role in the TME, currently deemed to be the worst culprit for cancer resistance.

Importantly, metformin can actually eradicate cancer stem cells, a pivotal aspect of cancer therapy, but conventional MTD ChT agents can hardly do so[46]. Moreover, MDSCs, being a main player of the TME[47], are also targeted by metformin[48,49]. For usually resistant cancers, *e.g.*, basal breast cancers, pre-clinical studies showed that a combination of metformin and a targeted therapy (erlotinib) could have encouraging results[50]. Thus, apart from observational and preclinical studies revealing metformin's activities on various cancers, it may now be worthwhile to undertake clinical trials (Figure 1). On the safety profile, despite being an anti-diabetic agent, hypoglycemia is hardly a significant problem, unlike most other anti-diabetics. Actually, over many decades, it has proven to be well tolerated and safe.

DISCUSSION

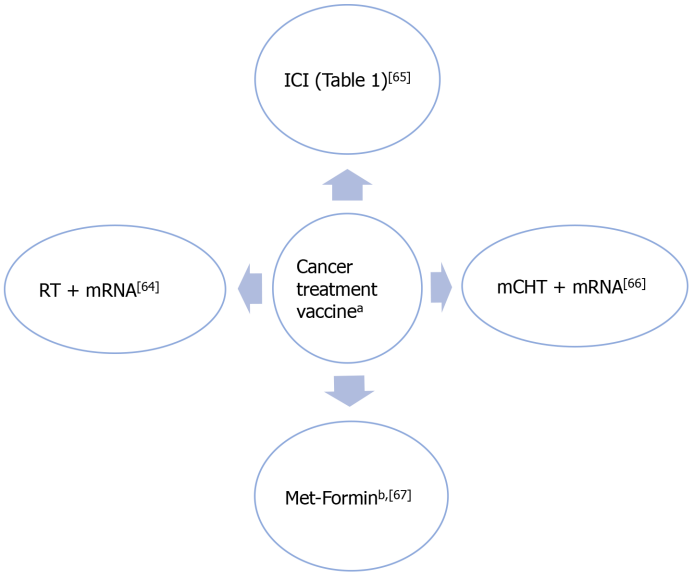
Although mRNA vaccines have already been tried clinically for oncology even two decades ago, the implementation for oncology has obviously been lagging behind. Actually, there has been significant technical advancements[51,52]. The current COVID-19 pandemic, though most disastrous, has ironically provided a good platform to highlight the safety profile of mRNA vaccines when the nucleoside-modified mRNA-LNP vaccines have a remarkable safety track record[53]. Actually, mRNA-LNPs can induce superior T follicular helper cell responses than that of an adjuvanted protein subunit vaccine even though the exact mechanism is still unclear. Moreover, although conventional pathogen vaccines usually require adjuvants to boost the much desired immunity, LNPs readily act as self-adjuvants[54]. Repeated oncology administrations would thus be facilitated, as there is hardly any issue of possible toxicity due to vaccine adjuvants typical of repurposed pathogen vaccines.

Importantly, mRNA vaccines represent a promising platform for the development of oncology vaccines as they can induce potent T cell responses and can also be readily modified[55]. Moreover, as mRNA vaccine design is highly flexible, it would enable the development of personalized neoantigen cancer vaccines, unless the cost becomes a significant concern, *e.g.*, during the current severe economic recession. As various aspects of novel developments, pivotal considerations, as well as current challenges for successful development of the self-amplifying RNA (saRNA) vaccines are already fully discussed elsewhere, suffice it to say that, even though the saRNA is very remarkable for enabling lower vaccine doses, the stability and manufacturing may still be challenging[17,56-59]. Encouragingly, it is now feasible to design very promptly a new saRNA vaccine for testing, as in the case of the Imperial College London[59]. The rapid and easy manufacture of saRNA vaccines could enable local productions so as to reduce logistical and cold-chain issues of current mRNA vaccines. Importantly, minimizing the required dose is highly desirable as it reduces side effects, *e.g.*, myocarditis and permits repeated usage for oncology practice.

Although testing of new modalities of oncology treatments often involve advanced cancers, the TME is actually very well known to be a major factor preventing successful testing of treatment options designed to cater for advanced cancers[60]. It would be more appropriate to test clinically these novel agents without the interference of the TME. For instance, for advanced melanomas, a recent randomized phase II clinical trial was on the efficacy of autologous DCs co-electroporated with mRNA coding for TriMix as well as mRNA encoding one of four TAAs linked to one HLA class II targeting signal (TriMixDC-MEL) (see Table 1)[61]. The randomization involved 41 patients (21 receiving TriMixDC-MEL; 20 had placebo). All patients had stage III/IV melanomas but no evidence of any residual disease (after resecting all macro-metastases). The vaccine was found to be tolerable and the 1-year disease free survival rate was 71% for the TriMixDC-MEL arm *vs* 35% of the placebo arm[61]. Admittedly, although not all melanoma metastases could likewise be resected, this trial would still demonstrate the vaccine's tolerability and probable effectiveness. This could not have been accomplished had the trial been performed on patients with significant TMEs.

CONCLUSION

The future development of mRNA vaccines for oncology is two pronged. On the one hand, as neoantigens of cancer cells are often dissimilar among individual patients, personalized vaccines are most appropriate, *e.g.*, the intranodal vaccine injection with free mRNA encoding 10 neoepitopes on 13 advanced melanoma patients could generate T cell immunity against multiple neoepitopes in all 13 patients[56,62]. Several personalized cancer vaccines using lipid nanoparticle–mRNA formulations have



DOI: 10.5306/wjco.v13.i8.663

Figure 1 Selected combinations with cancer treatment vaccines: Immune checkpoint inhibitors, radiotherapy, metronomic chemotherapy, and metformin. [a]Especially mRNA cancer vaccines: cell-free, rapid production, versatile and inherent adjuvant properties outperforming pathogen vaccines repurposed for oncology. Even balancing innate and adaptive immunities is feasible with mRNA. [b]Metformin's long standing safety track record, ready availability and eminent affordability may enable an ideal combination with mRNA cancer vaccines. ICI: Immune checkpoint inhibitors; mChT: Metronomic chemotherapy; RT: Radiotherapy.

also entered clinical trials, *e.g.*, mRNA-4157 is being tried actively both as monotherapy and in combination with ICIs (see Table 1).

On the other hand, such most impressive personalized oncology treatments, though much more specific, probably effective, and now with reduced processing time than other personalized vaccines, may not be readily affordable for the vast majority of cancer patients especially at this very trying period of severe economic recession. Therefore, for priming tumors having highly evasive TMEs, combination chemotherapy, radiation, and vaccines may have better efficacy[63]. As there may even be a highly beneficial mutual enhancement effect of turning "cold" tumors into "hot" ones[8,40], it really pays to explore further by performing robust clinical trials to document if such combinations have the potential of being a more versatile approach.

FOOTNOTES

Author contributions: Tsao SY designed the plan of the article, performed the reference search, wrote the manuscript, and has read and approved the final manuscript.

Conflict-of-interest statement: No conflict of interest is declared.

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S-Editor: Wang LL
L-Editor: Wang TQ
P-Editor: Wang LL

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World Journal of Clinical Oncology

Submit a Manuscript: https://www.f6publishing.com

World J Clin Oncol 2022 August 24; 13(8): 675-688

DOI: 10.5306/wjco.v13.i8.675

ISSN 2218-4333 (online)

ORIGINAL ARTICLE

Clinical and Translational Research

Eight hub genes as potential biomarkers for breast cancer diagnosis and prognosis: A TCGA-based study

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Specialty type: Oncology

Provenance and peer review: Unsolicited article; externally peer reviewed.

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

P-Reviewer: Kao TJ, Taiwan; Nakayama J, Japan

Received: August 2, 2021
Peer-review started: August 2, 2021
First decision: November 6, 2021
Revised: December 23, 2021
Accepted: July 26, 2022
Article in press: July 26, 2022
Published online: August 24, 2022

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Abstract

BACKGROUND

Breast cancer (BC) is the most common malignant tumor in women.

AIM

To investigate BC-associated hub genes to obtain a better understanding of BC tumorigenesis.

METHODS

In total, 1203 BC samples were downloaded from The Cancer Genome Atlas database, which included 113 normal samples and 1090 tumor samples. The limma package of R software was used to analyze the differentially expressed genes (DEGs) in tumor tissues compared with normal tissues. The cluster Profiler package was used to perform Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of upregulated and downregulated genes. Univariate Cox regression was conducted to explore the DEGs with statistical significance. Protein-protein interaction (PPI) network analysis was employed to investigate the hub genes using the CytoHubba plug-in of Cytoscape software. Survival analyses of the hub genes were carried out using the Kaplan-Meier method. The expression level of these hub genes was validated in the Gene Expression Profiling Interactive Analysis database and Human Protein Atlas database.

RESULTS

A total of 1317 DEGs (fold change > 2; $P < 0.01$) were confirmed through bioinformatics analysis, which included 744 upregulated and 573 downregulated genes in BC samples. KEGG enrichment analysis indicated that the upregulated genes were mainly enriched in the cytokine-cytokine receptor interaction, cell

cycle, and the p53 signaling pathway ($P < 0.01$); and the downregulated genes were mainly enriched in the cytokine-cytokine receptor interaction, peroxisome proliferator-activated receptor signaling pathway, and AMP-activated protein kinase signaling pathway ($P < 0.01$).

CONCLUSION

In view of the results of PPI analysis, which were verified by survival and expression analyses, we conclude that *MAD2L1*, *PLK1*, *SAA1*, *CCNB1*, *SHCBP1*, *KIF4A*, *ANLN*, and *ERCC6L* may act as biomarkers for the diagnosis and prognosis in BC patients.

Key Words: Breast cancer; Bioinformatics; Hub gene; The Cancer Genome Atlas; Protein-protein interaction

Core Tip: This study identified 1317 DEGs related to the occurrence and development of breast cancer (BC), 165 DEGs related to prognosis, and 8 hub genes (*MAD2L1*, *PLK1*, *SAA1*, *CCNB1*, *SHCBP1*, *KIF4A* , *ANLN*, and *ERCC6L*). Each of these eight hub genes has different expression levels in BC and is significantly related to prognosis. The results of this study indicate that studying these DEGs may help provide a full understanding of the molecular mechanisms underlying BC pathogenesis and progression. Moreover, these hub genes may serve as potential prognostic markers and therapeutic targets, which provide a reference for more in-depth and extensive prospective clinical research.

Citation: Liu N, Zhang GD, Bai P, Su L, Tian H, He M. Eight hub genes as potential biomarkers for breast cancer diagnosis and prognosis: A TCGA-based study. *World J Clin Oncol* 2022; 13(8): 675-688
URL: https://www.wjgnet.com/2218-4333/full/v13/i8/675.htm
DOI: https://dx.doi.org/10.5306/wjco.v13.i8.675

INTRODUCTION

Breast cancer (BC) is the most common malignant tumor in women. In 2019, 268600 new BC patients and 41760 new BC deaths were reported, accounting for 30% of all new cancer cases and 15% of cancer-related deaths, respectively. The mortality of BC is second only to lung cancer[1]. In recent years, BC outcome has significantly improved and treatment strategies such as surgery, chemotherapy, radiotherapy, endocrine therapy, and targeted therapy have achieved fine clinical benefits[2], whereas patients with distant metastases are almost incurable[3]. In addition, even after resection of the primary tumor, 30% of early BC is prone to recurrence in distant organs[4]. In clinical practice, the treatment and prognosis of different molecular subtypes of BC are significantly different: estrogen receptor-positive (ER+) patients prefer endocrine therapy, human epidermal growth factor receptor 2-positive (HER2+) patients prefer targeted therapy, and poorly differentiated tumors are usually associated with a poor prognosis[5-7].

Recent studies have found that the occurrence and development of BC are related to many molecular markers. For example, the expression of cluster of differentiation 82 is significantly decreased in BC and is associated with disease progression and metastasis[8]. In addition, a study on triple-negative BC suggested that multiple long noncoding RNAs are associated with prognosis, including MAGI2-AS3, GGTA1P, NAP1L2, CRABP2, SYNPO2, MKI67, and COL4A6[9]. Advances in microarray and high-throughput sequencing technology provide strong support for the development of more reliable prognostic markers[10,11]. Genome wide expression profiling can reveal molecular changes in the process of tumorigenesis and development, and has proven to be an efficient method to identify key genes[12]. Therefore, it is particularly important to explore more sensitive and specific biomarkers to further understand the pathogenesis of BC and the choice of treatment strategies.

This public database-based study explored potential hub genes in the occurrence and development of BC through bioinformatics analysis of the gene expression profile and clinical characteristics of BC, in order to provide new biological targets and directions for the clinical diagnosis and treatment of BC.

MATERIALS AND METHODS

Data sources and processing

The Cancer Genome Atlas (TCGA) database is a cancer research project established by the. National Cancer Institute and National Human Genome Research Institute. It aims to understand the mechanism

of carcinogenesis and development of cancer cells and develop new diagnosis and treatment methods by collecting various types of cancer-related omics data. In this study, 1203 breast samples (fragments per kilobase million [FPKM] format) were downloaded from TCGA database (https://portal.gdc.cancer.gov/), including 1090 tumor samples and 113 normal samples. For a more accurate comparison of gene expression, FPKM data were converted to transcripts per million (TPM). At the same time, 1097 tumor samples containing clinical information were downloaded, and the data that did not match the expression samples were excluded. The remaining 1089 tumor samples were included in the univariate Cox regression analysis. Overall survival (OS) was taken as the endpoint event, and gene expression in TPM format was converted to $\log_2 (x + 1)$.

DEG acquisition

Limma package of R software (version 3.6.3) was employed for differential gene analysis[13], using the adjusted *P*-value (adj *P*-value) to avoid false-positive results. The inclusion criteria of DEGs were: | log2 fold change (FC) | > 2 and adjusted $P < 0.01$. The ggplot2 package of R software was used to generate a volcano plot to visualize these differential genes.

Functional enrichment analysis

DEGs were converted into gene ID through org.Hs.eg.db package of R software, and then Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was carried out by R software's clusterProfiler and enrichlot program package. ggplot2 program package was used to display the top 10 enrichment items, and adjusted $P < 0.05$ was considered statistically significant.

Univariate Cox regression analysis

The survival package of R software was used to carry out univariate Cox regression analysis on 1089 BC samples with survival information. The median value of expression was set as the cut-off point between the high expression and low expression groups, and differential genes related to prognosis were obtained for subsequent analysis. $P < 0.05$ was considered statistically significant.

Construction of PPI

The STRING database (https://string-db.org/) is a search tool for searching interacting genes, which aims to construct protein-protein interaction (PPI) networks of different genes based on known and predicted PPIs, and analyze the proteins that interact with each other[14]. Based on the online tool STRING, PPI of prognosis-related DEGs was constructed, and the confidence score was ≥ 0.4. Then the PPI network was visualized by Cytoscape software (version 3.7.2). In addition, using the CytoHubba plug-in of Cytoscape software to calculate the gene degree through the "degree" method, the top 10 genes were taken as the hub genes for subsequent analysis and verification.

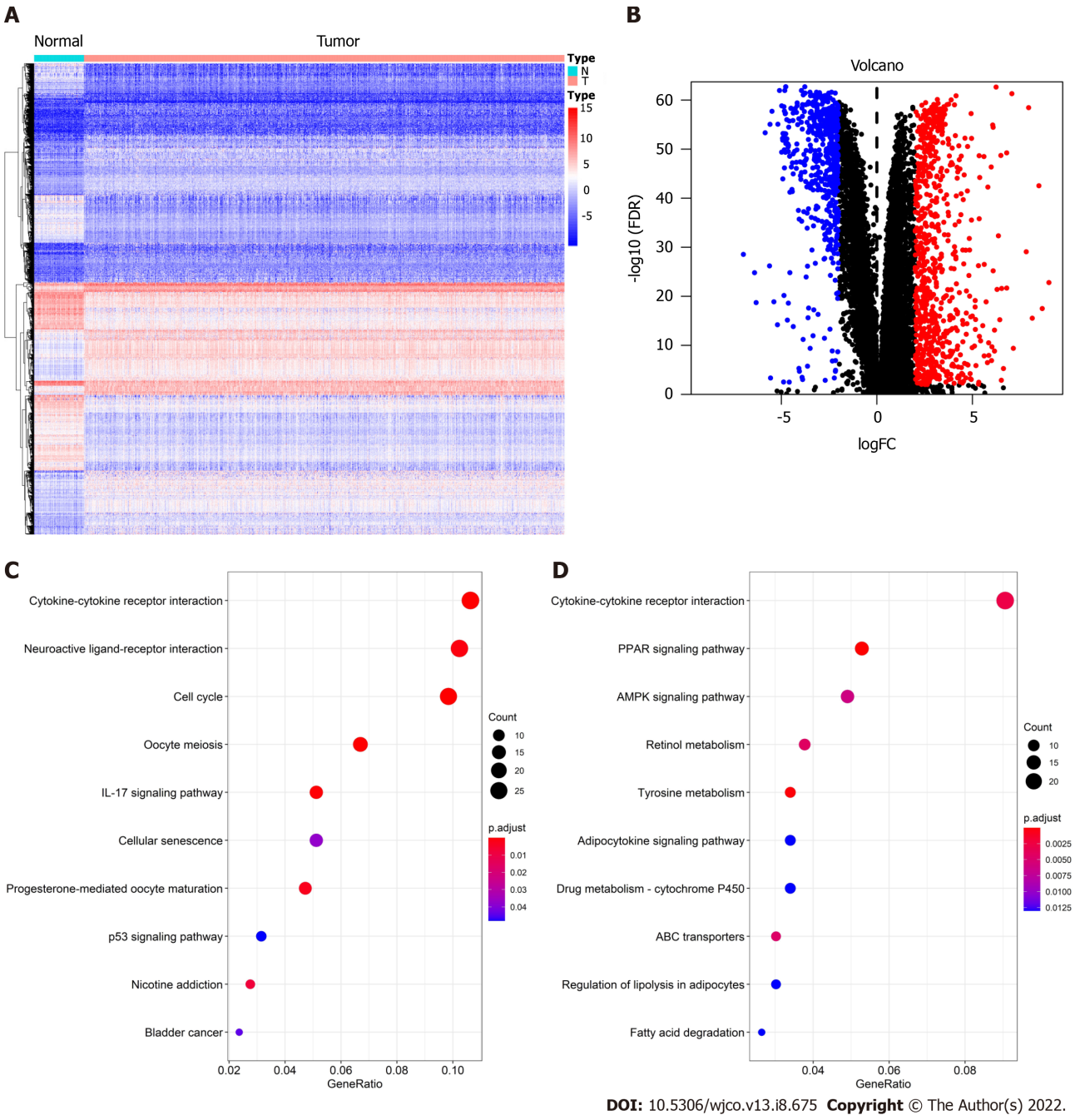
Survival analysis of hub genes

The Kaplan-Meier plotter (http://kmplot.com/analysis/) can use 18674 cancer samples to evaluate the impact of 54675 genes on survival[15]. These studies included recurrence-free survival and OS information of 5143 cases of BC, 1816 cases of ovarian cancer, 2437 cases of lung cancer, 1065 cases of gastric cancer, and 364 cases of liver cancer, which are mainly based on Gene Expression Omnibus, TCGA, and European Genome-phenome Archive databases. The role of the tool is to benefit patients in clinical decision making, health care policy, and resource allocation through meta-analysis of biomarker assessment[16]. In this study, we analyzed the OS rate of 10 hub genes in BC using the Kaplan-Meier plotter. According to the median expression of each hub gene in Kaplan-Meier plotter, the patients were divided into two groups to present the difference in survival probability between the high expression group and the low expression group. A total of 14 datasets were enrolled in our analysis according to the Kaplan-Meier web tool and detailed retrospective clinical information in http://kmplot.com/analysis/. $P < 0.05$ was considered statistically significant.

To further investigate the prognostic value of the hub genes selected above, we performed the log-rank test on these hub genes in molecular subtypes of BC based on TCGA cohort. Through the PAM50 algorithm, TCGA cohort was separated into five major subtypes: luminal A, luminal B, HER2 enriched, basal-like, and normal-like. This method was completed through utilizing the "genefu" R package according to detailed operation protocol.

Expression analysis of hub genes

The Gene Expression Profiling Interactive Analysis (GEPIA) database was employed to verify the mRNA expression levels of 10 hub genes in normal breast tissues and cancer tissues. GEPIA database contains data from 9736 tumor samples and 8587 normal samples, which were used to display the mRNA expression levels of each key gene in cancer and non-cancer tissues[17]. The protein expression levels of 10 hub genes in human normal tissues and BC tissues were analyzed using the human protein atlas database (HPA), which contains immunohistochemical expression data covering about 20 of the most common types of cancer[18].



DOI: 10.5306/wjco.v13.i8.675

Figure 1 Screening and functional enrichment analysis of differentially expressed genes. A: Heat map of differentially expressed genes (DEGs); B: Volcano Plot of DEGs; C: Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of upregulated genes; D: KEGG enrichment analysis of downregulated genes.

RESULTS

Identification and functional analysis of DEGs

After DEG analysis of 113 normal breast samples and 1090 BC samples, we found that there were 1317 DEGs, of which 744 were upregulated and 573 were downregulated in BC. As shown in Figure 1A, red represents high expression and blue represents low expression. At the same time, the volcano plot was used to present the distribution of DEGs (Figure 1B), the red dots represent upregulated genes and the blue dots represent downregulated genes.

To further understand the biological function of these 1317 DEGs, the clusterProfiler and enrichplot packages of R software were used to perform KEGG enrichment analysis on these DEGs. The enrichment analysis results of upregulated genes and downregulated genes are shown in Figure 1C and D, respectively. The top 10 upregulated genes were the cytokine-cytokine receptor interaction, neuroactive ligand-receptor interaction, cell cycle, oocyte meiosis, interleukin 17 signaling pathway, cellular senescence, progesterone-mediated oocyte maturation, p53 signaling pathway, nicotine

Table 1 Summary of the top 10 hub genes according to their grade

Genes	Gene name	Grade
MAD2L1	MAD2 mitotic arrest deficient-like 1	24
PLK1	Polo-like kinase 1	22
SAA1	Serum amyloid A1	22
CCNB1	Cyclin B1	20
SHCBP1	SHC SH2-domain binding protein 1	18
KIF4A	Kinesin family member 4A	18
ANLN	Actin binding protein	16
ERCC6L	Excision repair cross-complementation group 6-like	16
CXCL2	Chemokine (C-X-C motif) ligand 2	16
WT1	Wilms tumor 1	14

addiction, and bladder cancer. The 10 ten downregulated genes were the cytokine-cytokine receptor interaction, peroxisome proliferator-activated receptor (PPAR) signaling pathway, AMP-activated protein kinase (AMPK) signaling pathway, retinol metabolism, tyrosine metabolism, adipocytokine signaling pathway, drug metabolism - cytochrome p450, ATP-binding cassette transporters, regulation of lipolysis in adipocytes, and fatty acid degradation.

Screening of hub genes

To screen the DEGs related to the prognosis of BC, we used the survival package of R software to perform univariate Cox regression analysis on 1317 DEGs, and found that the prognosis of 165 genes was statistically significant (Supplementary Table 1). As shown in Figure 2, further analysis of the PPI of these 165 genes revealed that there were a total of 164 nodes and 156 interactions (edges), and the confidence score adopted default value ≥ 0.4. The CytoHubba algorithm of Cytoscape software was used to calculate the degree score of each node. The top 10 genes were MAD2L1, PLK1, SAA1, CCNB1, SHCBP1, KIF4A, ANLN, ERCC6L, CXCL2, and WT1 (Figure 3). The upregulated genes were represented by red and round nodes, and the downregulated genes were represented by blue and diamond nodes. The node size represented the level, and most of the hub genes were upregulated DEGs. Gene annotation and grade scores are shown in Table 1.

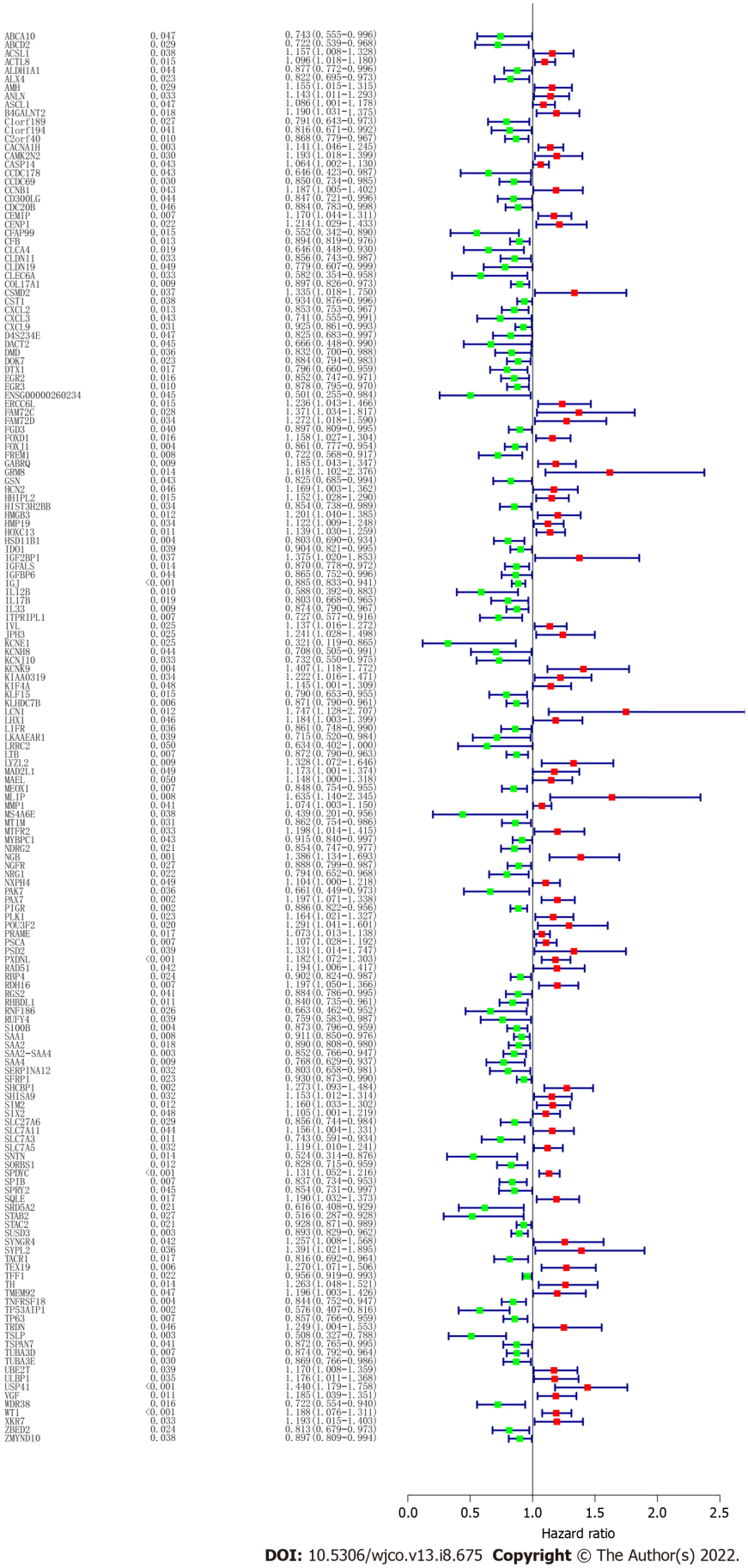
Survival analysis of hub genes

Kaplan-Meier plotter was used to explore the prognostic value of 10 hub genes in BC. The results showed that, except for *CXCL2* [hazard ratio (HR) 0.86 (0.69-1.07); $P = 0.170$] and *WT1* [HR 1.03 (0.83-1.28); $P = 0.760$], the highly expressed *MAD2L1* [HR 2.02 (1.62-2.51); $P = 1.8e-10$], *PLK1* [HR 1.42 (1.15-1.76); $P = 0.0012$], *CCNB1* [HR 1.42 (1.04-1.94); $P = 0.028$], *SHCBP1* [HR 1.76 (1.42-2.19); $P = 2.1$ e-07], *KIF4A* [HR 1.8 (1.44-2.23); $P = 8.8e-08$], *ANLN* [HR 1.48 (1.08-2.03); $P = 0.014$], and *ERCC6L* [HR 1.68 (1.35-2.09); $P = 2e-06$] were related to the poor OS rate of BC patients. By contrast, the high expression of *SAA1* [HR 0.71 (0.57-0.88); $P = 0.018$] was associated with a better OS rate for BC patients (Figure 4).

We also conducted the survival analysis of these 10 hub genes in TCGA molecular subtypes. As a result, TCGA cohort was successfully divided into five subtypes based PAM50 identifier: 563 of luminal A, 215 of luminal B, 82 of HER2-enriched, 189 of basal-like, and 39 of normal-like. Then survival analysis of these 10 genes was performed in each subtype group. The results indicated that *CXCL2* (HR = 0.45; $P < 0.05$) and *SAA1* (HR = 0.53; $P < 0.05$) were protective factors in the luminal A subtype (Figure 5). *ANLN* (HR = 2.12; $P < 0.05$), *ERCC6L* (HR = 3.04; $P < 0.05$), *KIF4A* (HR = 2.50; $P < 0.05$), *PLK1* (HR = 2.40; $P < 0.05$), and *SHCBP1* (HR = 2.42; $P < 0.05$) were hazard factors in luminal B subtype, whereas the *CXCL2* (HR = 0.45; $P < 0.05$) showed protective effects. Finally, *KIF4A* (HR = 4.31; $P < 0.05$) acted as a risk factor in *HER2*-enriched patients and *CXCL2* played a satisfactory role among basal-like patients (HR = 0.46; $P < 0.05$).

Expression analysis of hub genes

To verify the expression differences of key genes in BC, *GEPIA* was employed to analyze the mRNA expression levels of *MAD2L1*, *PLK1*, *SAA1*, *CCNB1*, *SHCBP1*, *KIF4A*, *ANLN*, *ERCC6L*, *CXCL2*, and *WT1* between BC and non-cancerous tissues (Figure 5). Compared with non-cancerous tissues, MAD2L1 (Figure 5A), PLK1 (Figure 5B), CCNB1 (Figure 5D), SHCBP1 (Figure 5E), KIF4A (Figure 5F), ANLN (Figure 5G), and ERCC6L (Figure 5H) in BC tissues were significantly upregulated ($P < 0.01$); SAA1 (Figure 5C) and CXCL2 (Figure 5I) were significantly downregulated in BC ($P < 0.01$); and WT1 (Figure 5J) tended to increase in BC tissues. After verifying the mRNA expression level of hub genes, we



DOI: 10.5306/wjco.v13.i8.675

Figure 2 Protein-protein interaction network analysis of prognosis related differentially expressed genes. The upregulated genes are

represented by red and round nodes, whereas the downregulated genes are represented by blue and diamond nodes. The size of the node represents their grade.

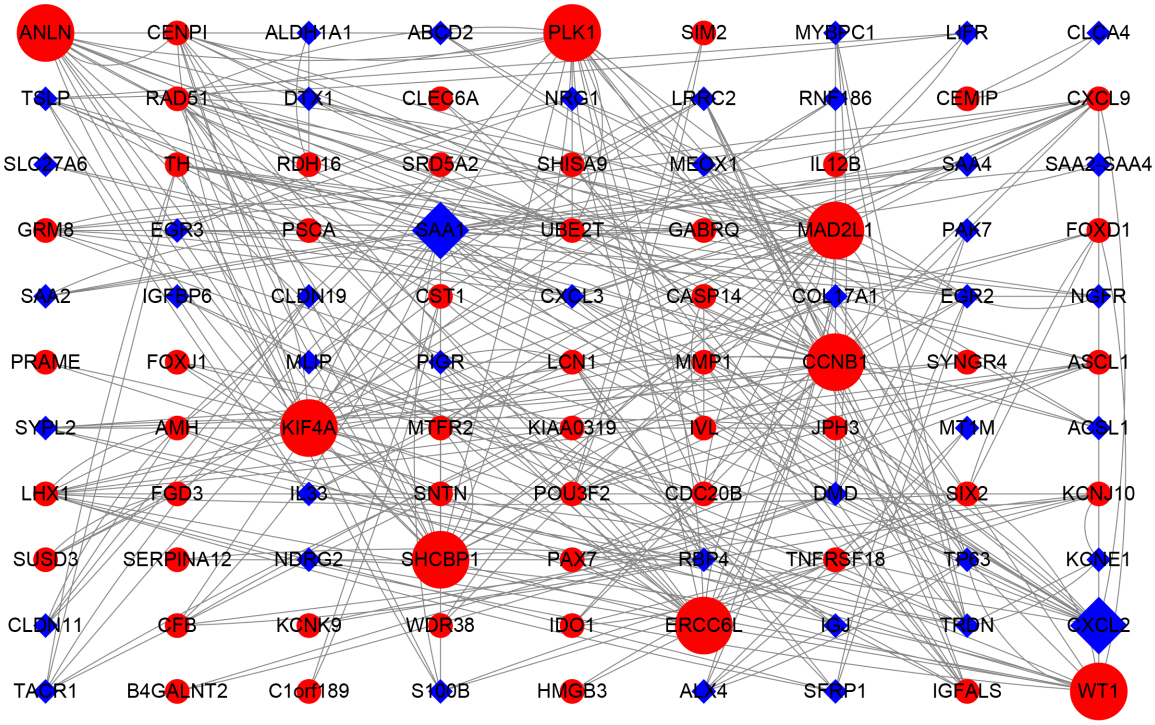


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Figure 3 Survival analyses of the 10 hub genes were verified by Kaplan-Meier plotter.

used the HPA database to verify the protein expression level of these hub genes in BC. It is worth noting that MAD2L1 (Figure 6A), PLK1 (Figure 6B), CCNB1 (Figure 6C), SHCBP1 (Figure 6D), ANLN (Figure 6F), ERCC6L (Figure 6G), and WT1 (Figure 6H) were not expressed in normal breast tissues, but expressed in different levels in BC tissues. KIF4A (Figure 6E) was moderately expressed in normal breast tissues and highly expressed in BC tissues. In short, the expression of hub genes was consistent with the results of differential analyses at both the mRNA and protein levels.

DISCUSSION

In this study, we used bioinformatics analysis to screen and verify potential biomarkers associated with BC. After comparing the gene expression matrix of breast tissue retrieved from TCGA database, 744 upregulated DEGs and 573 downregulated DEGs were successfully identified. Combined with the survival data, 165 prognostic-related DEGs were analyzed. According to PPI network analysis, the top 10 node genes were ranked: *MAD2L1*, *PLK1*, *SAA1*, *CCNB1*, *SHCBP1*, *KIF4A*, *ANLN*, *ERCC6L*, *CXCL2*, and *WT1*. After subsequent survival analysis and expression analysis verification, the expression and prognosis of *MAD2L1*, *PLK1*, *SAA1*, *CCNB1*, *SHCBP1*, *KIF4A*, *ANLN*, and *ERCC6L* in BC were finally confirmed. These eight hub genes may play a vital role in the occurrence and development of BC.

Among the 1317 identified DEGs, significant gene expression dysregulation was observed in the cell cycle, PPAR signaling pathway, and AMPK signaling pathway. Cell cycle is a highly conserved process in human evolution and is essential for the normal growth of cells. Abnormal cell cycle is a hallmark of human cancer[19]. Recent studies have also identified several genes related to the cell cycle, including *CCNB1*, *ANLN*, *MAD2L1*, and *PLK1*. For example, CCNB1 may be a biomarker for the prognosis of ER+ BC patients and monitoring the efficacy of hormone therapy[20]. Recent studies have found that the occurrence and proliferation of gastric cancer cells induced by ISL1 is mediated by the expression and regulation of CCNB1, CCNB2, and C-MYC[21]. In addition, the high expression of ANLN in BC cell nuclei is significantly related to tumor tissue size, histopathological grade, high proliferation rate, and a worse prognosis[22]. *MAD2L1* is a mitotic spindle checkpoint gene. In patients with primary BC, compared with patients with ER+, PR+ and low-grade tumors, patients with ER-, PR- and high-grade tumors have higher expression of MAD2L1, and high expression of MAD2L1 is associated with a poor OS[23]. PLK1 is a key oncogene that can regulate the transition of cells in the G2-M phase, thus

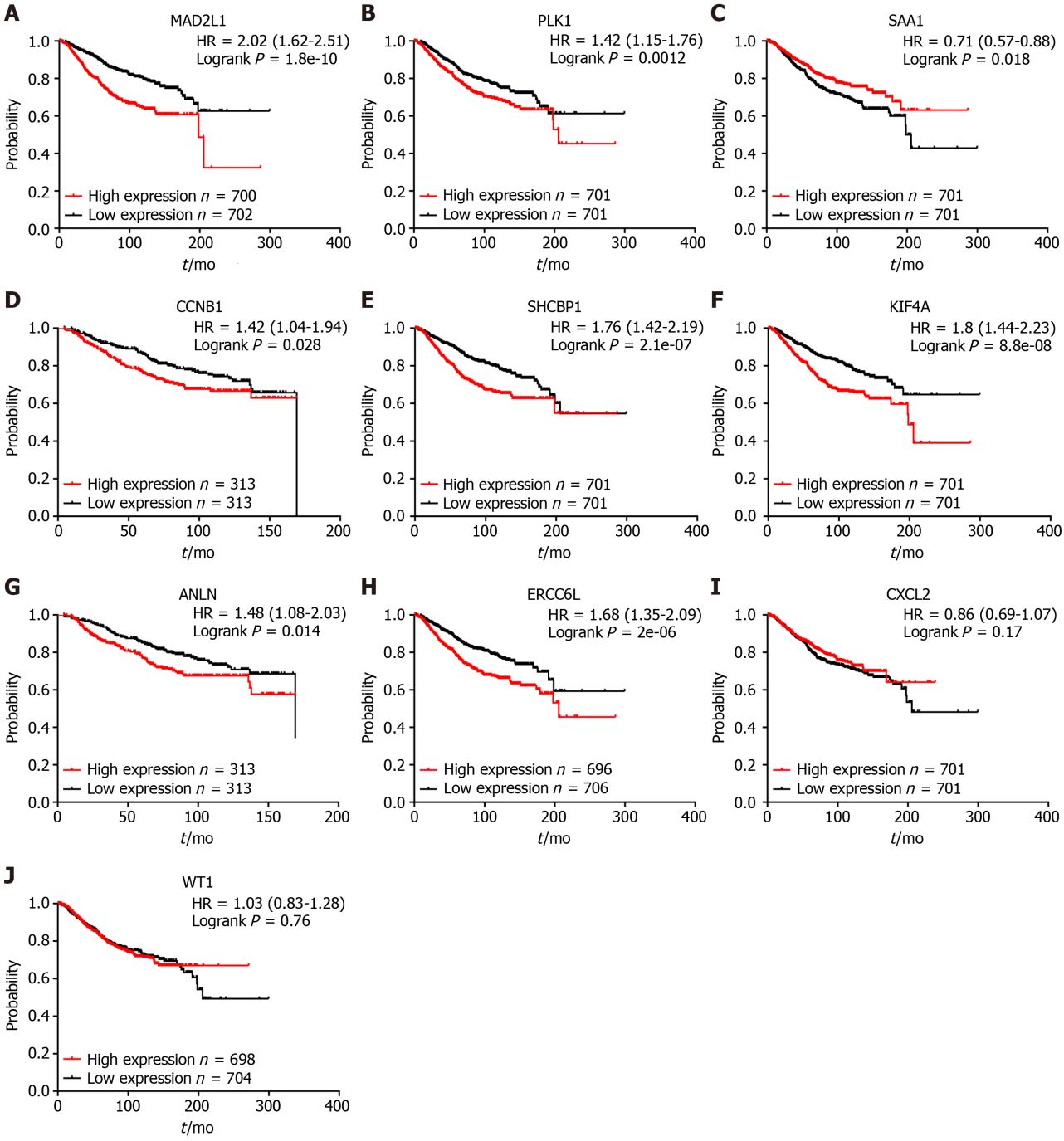

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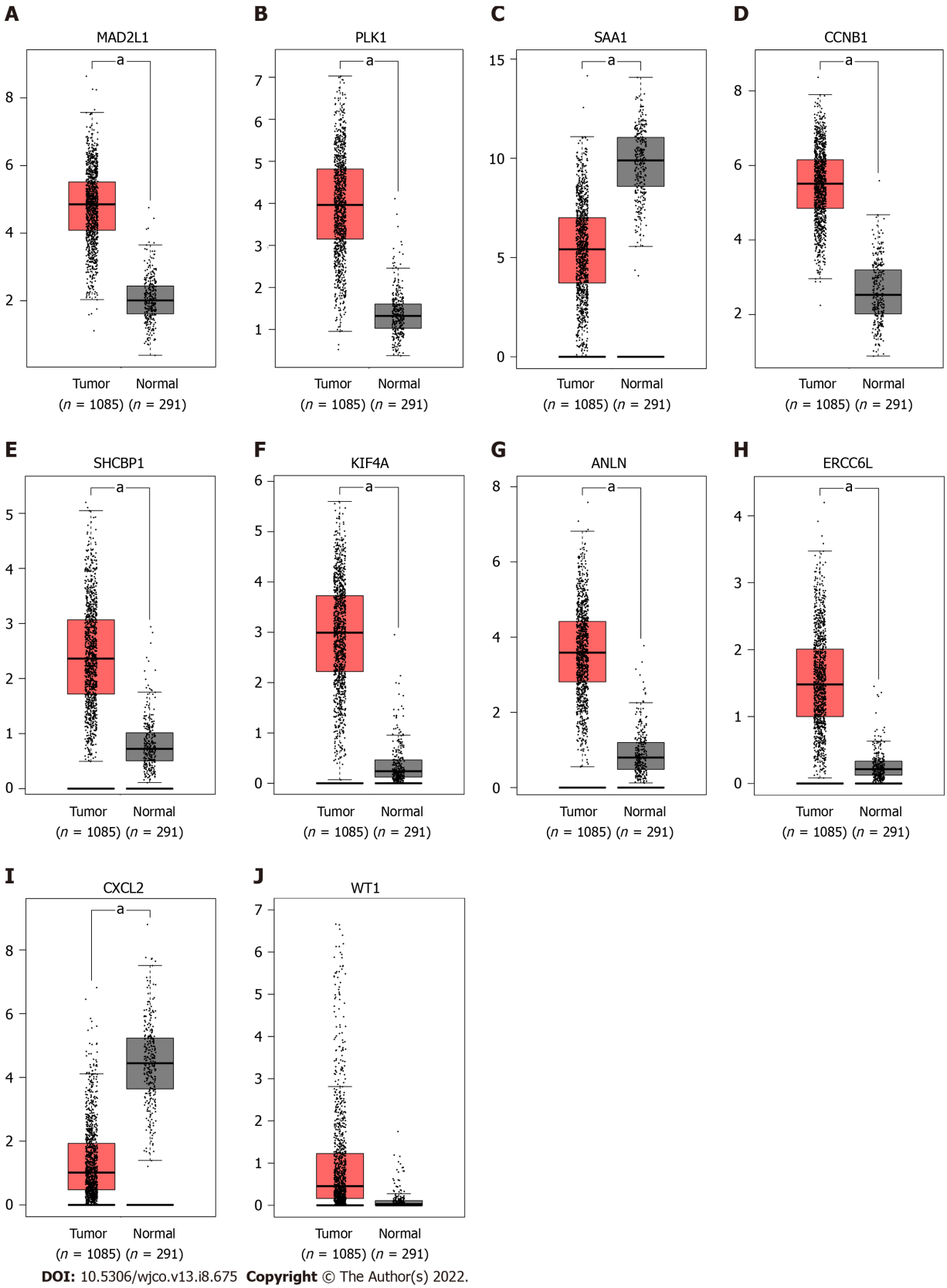
Figure 4 Subtype survival analysis of these 10 hub genes in breast cancer patients among The Cancer Genome Atlas cohort. The results are presented by a heatmap and the detailed value on each cell represent the hazard ratio of survival plot.

promoting the growth and metastasis of tamoxifen resistant BC[24]. These studies are consistent with our current conclusion that *CCNB1*, *ANLN*, *MAD2L1*, and *PLK1*, as key genes, are overexpressed in BC tissues, and their overexpression is correlated with poor prognosis. Meanwhile, the PPAR signaling pathway may be an important predictor of BC response to neoadjuvant chemotherapy[25], and activation of the AMPK signaling pathway can inhibit the activity of the Wnt/β-catenin signaling pathway, thereby inhibiting the growth of BC cells[26]. These studies showed that the identified DEGs play a critical role in the occurrence and development of BC, and the hub genes among them may serve as prognostic markers and are worth further investigation.

With the exception of *CCNB1*, *ANLN*, *MAD2L1*, and *PLK1*, the gene combination model of CD74, MMP9, RPA3, and SHCBP1 in the tumor microenvironment (TME) can effectively predict the prognosis and disease risk of BC patients[27], while their potential mechanism remains unknown. In addition, the circKIF4A-miR-375-KIF4A axis can regulate the development of triple-negative BC through competing endogenous RNA, and circKIF4A can act as a prognostic biomarker and therapeutic target for triple-

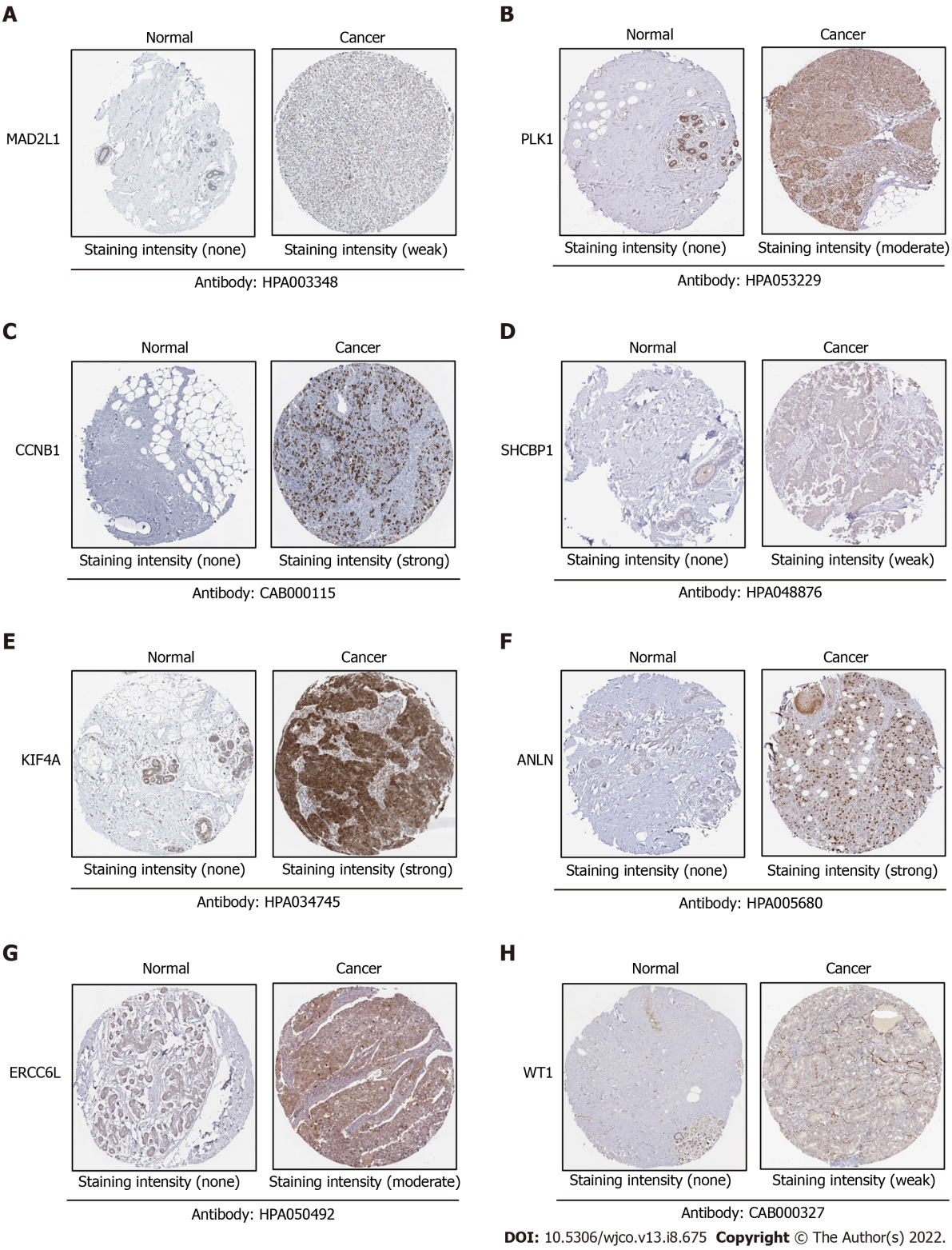


DOI: 10.5306/wjco.v13.i8.675

Figure 5 mRNA expression of the 10 hub genes were verified by the Gene Expression Profiling Interactive Analysis database. $^{a}P < 0.05$.

negative BC[28].

SAA1 is a serum amyloid protein family member that is highly expressed in non-small cell lung cancer, and is associated with a poor prognosis and tyrosine kinase inhibitors[29]. SAA1 has low expression in hepatocellular carcinoma, and the high expression of SAA1 is associated with a better prognosis[30]. To date, SAA1 has not been reported in BC, and the specific role and function of this gene



DOI: 10.5306/wjco.v13.i8.675

Figure 6 Protein expression of the eight hub genes were verified by human protein atlas database. The database lacks expression data on serum amyloid A1- and chemokine (C-X-C motif) ligand 2-related proteins.

in BC require further experimental exploration and clinical specimen verification. ERCC6L is a newly discovered DNA helicase. In the human BC cell line MDA-MB-231, exogenous interference with the expression of ERCC6L can inhibit the growth of BC cells[31]. However, its role and specific mechanism in clinical specimens are still unknown. The expression of ERCC6L is upregulated in clear cell renal cell carcinoma, and the highly expressed ERCC6L can promote the proliferation of clear cell renal cell carcinoma cells by regulating the mitogen-activated protein kinase signaling pathway[32]. In this study, we found that SAA1 and ERCC6L may be used as prognostic markers for BC, whereas there are few reports on these two genes, and further research is necessary.

In this study, we found that the differential expression of the eight hub genes are related to the occurrence and development of BC, and are significantly related to the OS rate, which indicate that these hub genes may be utilized as potential prognostic biomarkers and therapeutic targets for BC. This study had some limitations. First, due to the complexity of the dataset in the public database, it is difficult to consider some important confounding factors such as different ages, races, regions, and tumor stages when analyzing DEGs. Second, according to the results, seven key genes were upregulated in BC and one key gene was downregulated, but the mechanism of their differential expression is still unclear, and more studies are needed to confirm their biological basis. Finally, this study focused on the expression level and OS rate of the eight hub genes, and whether these key genes can be used as biomarkers and can improve the diagnostic accuracy and specificity of BC requires further research.

CONCLUSION

In conclusion, based on comprehensive bioinformatics analysis, this study identified 1317 DEGs related to the occurrence and development of BC, 165 DEGs related to prognosis, and 8 hub genes (*MAD2L1*, *PLK1*, *SAA1*, *CCNB1*, *SHCBP1*, *KIF4A*, *ANLN* and *ERCC6L*). Each of these eight hub genes has different expression levels in BC and is significantly related to prognosis. The results of this study indicate that studying these DEGs would help us have a deeper understanding of the molecular mechanisms of the pathogenesis and progression of BC. Moreover, these hub genes may serve as potential prognostic markers and therapeutic targets for BC, which provides a reference for more in-depth and extensive prospective clinical research.

ARTICLE HIGHLIGHTS

Research background

Breast cancer (BC) is the most common malignant tumor in women. In 2019, 268600 new BC patients and 41760 new BC deaths were reported, accounting for 30% of all new cancer cases and 15% of cancer-related deaths. Therefore, it is particularly important to explore more sensitive and specific biomarkers for further understanding the pathogenesis of BC and the choice of treatment strategies.

Research motivation

Exploring more valuable therapeutic targets would be helpful in treating with high efficacy.

Research objectives

This study aimed to identify novel biomarkers for BC.

Research methods

The limma package of R software and clusterProfiler package were used to analyze the differentially expressed genes (DEGs) in tumor tissues compared with the normal tissues, respectively. The protein-protein interaction network (PPI) analysis was used to investigate the hub-genes through cytohubba algorithm by the Cytoscape software. Survival analysis of the hub-genes were carried out through the Kaplan-Meier database. The expression level of these hub-genes was validated in the GEPIA database and the Human Protein Atlas database.

Research results

Upregulated genes mainly enriched in the cytokine-cytokine receptor interaction, cell cycle, and p53 signaling pathway ($P < 0.01$). The downregulated genes were mainly enriched in the cytokine-cytokine receptor interaction, peroxisome proliferator-activated receptor signaling pathway, and AMP-activated protein kinase signaling pathway ($P < 0.01$).

Research conclusions

MAD2L1, PLK1, SAA1, CCNB1, SHCBP1, KIF4A, ANLN, and *ERCC6L* may act as biomarkers for diagnosis and prognosis in BC patients.

Research perspectives

Proper validations must be made in future studies.

FOOTNOTES

Author contributions: Liu N performed the experiment and wrote the paper; Liu N, Zhang GD, and Bai P contributed to the bioinformatics analysis and figure preparation; Tian H and Su L modified the structure and language of the manuscript; He M and Tian H contributed to the conception and design of the study and the revisions of the manuscript; All authors have read and approved the final manuscript.

Institutional review board statement: Not applicable.

Conflict-of-interest statement: The authors have no conflicts of interest to declare.

Data sharing statement: No additional data are available.

Country/Territory of origin: China

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S-Editor: Gong ZM
L-Editor: Filipodia
P-Editor: Gong ZM

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World Journal of Clinical Oncology

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World J Clin Oncol 2022 August 24; 13(8): 688-701

DOI: 10.5306/wjco.v13.i8.688

ISSN 2218-4333 (online)

ORIGINAL ARTICLE

Retrospective Cohort Study

Propensity-matched analysis of patients with intrahepatic cholangiocarcinoma or mixed hepatocellular-cholangiocarcinoma and hepatocellular carcinoma undergoing a liver transplant

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Specialty type: Oncology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

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

P-Reviewer: Bellini MI, Italy; Imai Y, Japan; Kao JT, Taiwan; Nakano M, Japan; Park M, South Korea

Received: April 8, 2022
Peer-review started: April 8, 2022
First decision: May 12, 2022
Revised: June 14, 2022
Accepted: July 11, 2022
Article in press: July 11, 2022
Published online: August 24, 2022

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Abstract

BACKGROUND

Cholangiocarcinoma (CC) is a rare tumor that arises from the epithelium of the bile ducts. It is classified according to anatomic location as intrahepatic, perihilar, and distal. Intrahepatic CC (ICC) is rare in patients with cirrhosis due to causes other than primary sclerosing cholangitis. Mixed hepatocellular carcinoma-CC (HCC-CC) is a rare neoplasm that shows histologic findings of both HCC and ICC within the same tumor mass. Due to the difficulties in arriving at the correct diagnosis, patients eventually undergo liver transplantation (LT) with a presumptive

diagnosis of HCC on imaging when, in fact, they have ICC or HCC-CC.

AIM

To evaluate the outcomes of patients with intrahepatic cholangiocarcinoma or mixed hepatocellular-cholangiocarcinoma on pathological examination after liver transplant.

METHODS

Propensity score matching was used to analyze tumor recurrence (TR), overall mortality (OM), and recurrence-free survival (RFS) in LT recipients with pathologically confirmed ICC or HCC-CC matched 1:8 to those with HCC. Progression-free survival and overall mortality rates were computed with the Kaplan-Meier method using Cox regression for comparison.

RESULTS

Of 475 HCC LT recipients, 1.7% had the diagnosis of ICC and 1.5% of HCC-CC on pathological examination of the explant. LT recipients with ICC had higher TR (46% *vs* 11%; $P = 0.006$), higher OM (63% *vs* 23%; $P = 0.002$), and lower RFS (38% vs 89%; $P = 0.002$) than those with HCC when matched for pretransplant tumor characteristics, as well as higher TR (46% *vs* 23%; $P = 0.083$), higher OM (63% *vs* 35%; $P = 0.026$), and lower RFS (38% *vs* 59%; $P = 0.037$) when matched for posttransplant tumor characteristics. Two pairings were performed to compare the outcomes of LT recipients with HCC-CC *vs* HCC. There was no significant difference between the outcomes in either pairing.

CONCLUSION

Patients with ICC had worse outcomes than patients undergoing LT for HCC. The outcomes of patients with HCC-CC did not differ significantly from those of patients with HCC.

Key Words: Cholangiocarcinoma; Hepatocellular carcinoma; Liver; Prognosis; Recurrence; Survival analysis; Transplantation

Core Tip: This retrospective cohort study analyzes the outcomes of patients undergoing liver transplantation (LT) with a presumptive diagnosis of hepatocellular carcinoma (HCC) in which explant analysis identified that they actually had intrahepatic cholangiocarcinoma (ICC) or mixed hepatocellular cholangiocarcinoma (HCC-CC). Propensity score matching was used to analyze tumor recurrence, overall mortality, and recurrence-free survival in LT recipients with pathologically confirmed ICC or HCC-CC matched 1:8 to those with HCC. Patients with ICC have worse outcomes than patients undergoing LT for HCC, even when matched for explant pathology. Outcomes did not differ significantly between patients with HCC-CC and patients with HCC.

Citation: Brandão ABM, Rodriguez S, Fleck Jr AM, Marroni CA, Wagner MB, Hörbe A, Fernandes MV, Cerski CT, Coral GP. Propensity-matched analysis of patients with intrahepatic cholangiocarcinoma or mixed hepatocellular-cholangiocarcinoma and hepatocellular carcinoma undergoing a liver transplant. *World J Clin Oncol* 2022; 13(8): 688-701

URL: https://www.wjgnet.com/2218-4333/full/v13/i8/688.htm

DOI: https://dx.doi.org/10.5306/wjco.v13.i8.688

INTRODUCTION

Cholangiocarcinoma (CC) is a relatively rare, aggressive tumor that arises from the epithelium of the bile ducts. It is classified according to anatomical location as intrahepatic, perihilar, or distal[1]. CC is the most common tumor of the biliary tree, accounting for approximately 10%-25% of all hepatic malignancies[2]. It is the second most common hepatic malignancy[3].

Intrahepatic CC (ICC) represents 5%-10% of all CCs[1,4,5]. Although rare, its incidence is increasing in many countries[6-9]. In Brazil, ICC-related mortality in persons aged 45-64 years increased by 100% from 2002 to 2012, reaching 0.35 and 0.37 per 100000 person-years for men and women, respectively[9]. The increase is attributed, at least in part, to improved ICC classification, accurate diagnosis, and the negative impact of known risk factors, such as chronic hepatitis C virus (HCV) infection and obesity[10].

Mixed hepatocellular-cholangiocarcinoma (HCC-CC) is a rare neoplasm that histologically resembles both HCC and ICC within the same tumor mass[11]. It has an estimated incidence of 1%-4.7% among hepatic malignancies[12]. HCC-CC and ICC share the same risk factors[13]. The diagnosis of HCC-CC is typically made by pathology after resection or transplant, and a preoperative diagnosis is unlikely[14].

Although imaging findings suggestive of the diagnosis of HCC, ICC, or HCC-CC have been described[15-17], these tumors can be challenging to diagnose because of their rarity. In addition, HCC and ICC can coexist in separate nodules within the same liver or within the same tumor mass. Therefore, due to the difficulties in arriving at the correct diagnosis, patients eventually undergo a liver transplant (LT) with the presumptive imaging diagnosis of HCC when, in fact, they have ICC or HCC-CC[18,19].

The present study aimed to determine the prevalence of ICC or HCC-CC confirmed by explant pathology in patients who underwent LT with the presumptive diagnosis of HCC and to compare recurrence, recurrence-free survival, and overall mortality rates between these patients and LT recipients with HCC.

MATERIALS AND METHODS

Study design and population

This retrospective cohort study included patients aged ≥ 18 years with liver cirrhosis and imaging findings suggestive of HCC within the Milan criteria who underwent LT between June 1997 and July 2019 at a transplant referral center/teaching hospital in southern Brazil. Patients were followed up until April 2020 and divided into three groups according to the diagnosis on explant pathology: (1) Patients with HCC; (2) Patients with ICC; and (3) Patients with mixed HCC-CC. Well-established diagnostic criteria were followed, and immunohistochemical analysis was performed if necessary[12,20].

The following variables were analyzed: Age, sex, etiology of liver cirrhosis, Child-Pugh score, pretransplant tumor characteristics, including presence and type of neoadjuvant therapy, highest alpha-fetoprotein (AFP) level, and sum of nodule diameters on imaging; and posttransplant characteristics (explant), including number of nodules and sum of nodule diameters, cases within the Milan criteria or University of California San Francisco (UCSF) criteria, tumor grade/differentiation, presence of total necrosis, and microvascular invasion.

The outcomes analyzed were tumor recurrence, recurrence-free survival, and overall mortality.

Brazilian criterion for inclusion of patients with HCC in the transplant waiting list

In Brazil, patients with liver cirrhosis and imaging findings suggestive of HCC[21,22] can be placed on the LT waiting list upon detection of a lesion ≥ 2 cm and ≤ 5 cm or up to three lesions ≥ 2 cm and ≤ 3 cm.

Pretransplant locoregional therapy

Patients on the waiting list with an estimated waiting time for LT > 6 mo were treated with transarterial chemoembolization, radiofrequency ablation, or percutaneous ethanol injection.

Statistical analysis

The statistical methods of this study were reviewed by Mario B. Wagner, MD PhD DLSHTM, Full Professor of Epidemiology and Biostatistics, School of Medicine, Federal University of Rio Grande do Sul, Brazil.

Baseline patient characteristics were described using standard statistical methods. Continuous variables were compared using *t*-test or Mann-Whitney test when distributional assumptions were in doubt. Categorical variables were compared by the chi-square test or Fisher's exact test when needed. Propensity score matching (PSM) was used to assess whether tumor recurrence, overall mortality, and recurrence-free survival rates in patients with ICC or HCC-CC differed from those in patients with HCC. Additionally, hazard ratios (HRs) and their confidence intervals (CIs) were calculated. Progression-free survival rate and overall mortality rate were computed with the Kaplan-Meier method using Cox regression for comparison.

Propensity score matching

Patients with ICC and HCC-CC were matched to those with HCC using PSM based on the nearest neighbor algorithm according to a 1:8 ratio. Considering pretransplant and posttransplant variables, two matching sequences were run for patients with ICC and another two sequences for those with HCC-CC, which resulted in four matching datasets.

The variables considered for the pretransplant matching were highest AFP level, largest nodule diameter or the sum of the largest diameters in the case of multiple lesions, and year of LT. The posttransplant matching was based on variables collected during explant pathology which included tumor grade/differentiation, microvascular invasion, largest nodule diameter or the sum of the largest diameters in the case of multiple lesions, and year of LT.

Simple Cox regression was applied to the four datasets (pretransplant variable-matched sets ICC *vs* HCC and HCC-CC *vs* HCC, and posttransplant variable-matched sets ICC *vs* HCC and HCC-CC *vs* HCC) to obtain HRs and 95%CIs.

PSM groups were defined using R version 4.0 and the package MatchIT (software package MatchIT in R version 4.0.4; https://www.r-project.org/). Other analyses were conducted with IBM-SPSS version 25. *P* values < 0.05 were considered statistically significant.

Ethical aspects

The study followed the guidelines for the publication of observational studies[23]. The Institutional Review Board of Santa Casa de Misericórdia de Porto Alegre approved the study protocol (No. 4.250.889). Informed consent was waived due to the non-interventional design of the study and retrospective nature of data collection. All investigators signed a data use agreement to ensure the ethical and secure use of the data.

RESULTS

Over a period of 22 years, 475 patients with the presumptive diagnosis of HCC underwent LT at our center. According to a retrospective review of the LT database, 15 of these patients (3.1%) were found to have either ICC (n = 8) or HCC-CC (n = 7) detected in the pathological examination of the explant. The remaining 460 patients had the diagnosis of HCC confirmed by explant pathology (Figure 1). Most ICCs (6/8; 75.0%) were moderately or poorly differentiated and had the largest nodule diameter or the sum of the largest diameters < 5 cm. The patients with HCC-CC (7/7; 100%) were also moderately or poorly differentiated. In most HCC-CC cases (5/7; 71.4%), the largest nodule diameter or the sum of the largest diameters did not exceed 5 cm.

Comparison of ICC vs HCC transplant recipients, propensity score-matched for year of transplant and pretransplant and posttransplant tumor characteristics

Table 1 shows the comparison of patients with ICC (n = 8) matched 1:8 to those with HCC (n = 64) who underwent LT in the same year and had similar pretransplant tumor characteristics (median highest AFP level and cumulative radiologic tumor diameter). Demographic characteristics and mean age did not differ significantly between the two groups: most patients were men and the most common etiology of liver cirrhosis was HCV infection. The median highest AFP level of patients with ICC was higher than that of patients with HCC, although without statistical significance. Patients with ICC more commonly received bridging therapy for transplant (100% *vs* 67.2%; P = 0.036), but they were less responsive than patients with HCC (total necrosis: 12.5% *vs* 58.1%; P = 0.008). Also, according to explant pathology, patients with ICC had less differentiated tumors (grade 2 + 3: 75% *vs* 56.2%; P = 0.022) and higher rates of microvascular invasion (37.5% *vs* 9.4%; P = 0.056) (Table 1).

Figure 2 shows the risk of tumor recurrence, overall mortality, and recurrence-free survival. When comparing these risks between patients with ICC and HCC matched for pretransplant tumor characteristics, estimated by the simple Cox regression model, patients with ICC had a higher 3-year risk of recurrence (46% *vs* 11%; HR 7.14 [95%CI, 1.77-28.85]; P = 0.006) and overall mortality (63% *vs* 23%; HR 4.41 [95%CI, 1.72-11.32]; P = 0.002) and a lower recurrence-free survival rate (38% *vs* 77%; HR 4.42 [95%CI, 1.74-11.24]; P = 0.002).

Given the poorer outcomes of LT recipients with ICC and pretransplant tumor characteristics like those of LT recipients with HCC, we sought to assess whether these results would be explained by the potentially more aggressive nature of ICC. To this end, an additional PSM was performed by pairing patients with ICC and HCC with similar explant pathology (median cumulative tumor diameter, nuclear grade/differentiation, and microvascular invasion), but the groups did not differ significantly in these variables (Table 1). Compared with patients with HCC, those with ICC had a higher 3-year cumulative risk of tumor recurrence (46% *vs* 23%; HR 3.07 [95%CI, 0.86-10.94]; P = 0.083) and overall mortality (63% *vs* 35%; HR 2.78 [95%CI, 1.13-6.86]; P = 0.026) and a lower recurrence-free survival rate (38% *vs* 65%; HR 2.59 [95%CI, 1.06-6.31]; P = 0.037) (Figure 2).

Compared with HCC transplant recipients with similar pretransplant characteristics, patients with ICC had significantly higher 1- and 5-year overall mortality (62.5% and 81.2% vs 12.5% and 29.8%; P = 0.002) and lower 1- and 5-year RFS (37.5% and 18.8% *vs* 87.5% and 70.2%; P = -0.002). Compared with those with similar posttransplant characteristics (explant pathologic features), patients with ICC had significantly higher 1- and 5- year mortality (20.3% and 42.8% *vs* 12.5% and 29.8%; P = 0.002) and lower 1- and 5-year RFS (79.7% and 57.2% vs 87.5% and 70.2%; P = 0.002) (Figure 3).

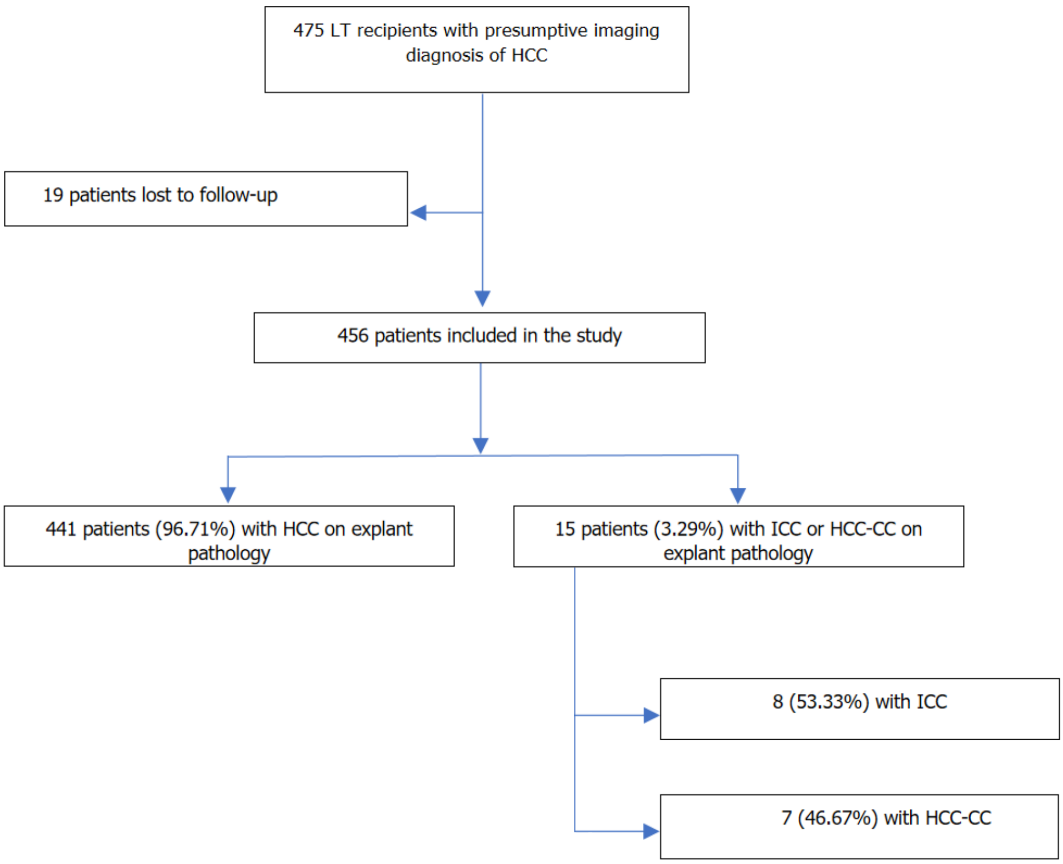
Comparison of HCC-CC vs HCC transplant recipients, propensity score-matched for year of transplant and pretransplant and posttransplant tumor characteristics

Two pairings were also performed, in a 1:8 ratio, between patients with HCC-CC (n = 7) and HCC (n = 56) who underwent LT in the same year. The first pairing considered similar pretransplant tumor

Table 1 Comparison of pretransplant tumor characteristics, locoregional therapy, and posttransplant tumor characteristics between patients with intrahepatic cholangiocarcinoma and hepatocellular carcinoma, matched 1:8 for pre-liver transplant factors and explant factors

Variable	Pre-LT factors			Explant factors	
	ICC (*n* = 8)	HCC (*n* = 64)	*P* value	HCC (*n* = 64)	*P* value
Recipient characteristics					
Age, mean ± SD	59.4 ± 7.6	61.5 ± 8.0	0.489	60.3 ± 8.6	0.774[2]
Male, *n* (%)	5 (62.5)	44 (68.8)	0.704	50 (78.1)	0.382[1]
Etiology of liver disease, *n* (%)			0.201		0.745[1]
HCV	6 (75.0)	50 (78.1)		45 (70.3)	
Alcohol	0	9 (14.1)		10 (15.6)	
HBV	0	1 (1.6)		3 (4.7)	
NAFLD	1 (12.5)	2 (3.1)		1 (1.6)	
Cryptogenic	1 (12.5)	1 (1.6)		2 (3.1)	
Other	0	1 (1.6)		3 (4.7)	
CTP class, *n* (%)			0.168		0.210[1]
A	7 (87.5)	43 (67.2)		40 (63.5)	
B	0	17 (26.6)		19 (30.2)	
C	1 (12.5)	4 (6.3)		4 (6.3)	
Maximum pretransplant AFP, ng/mL	28.5 (1.60-801.0)	10.8 (1.7-1133.0)	0.324	12.5 (1.3-6123.0)	0.620[3]
Radiographic tumor characteristics					
Cumulative tumor diameter, cm, *n* (%)			0.072		0.862[1]
< 2.1	1 (12.5)	21 (32.8)		9 (14.1)	
2.2-5.0	4 (50.0)	37 (57.8)		39 (60.9)	
> 5.1	3 (37.5)	6 (9.4)		16 (25.0)	
Neoadjuvant therapy, *n* (%)			0.036		0.016[1]
None	0	21 (32.8)		21 (32.8)	
TACE	8 (100.0)	32 (50.0)		29 (45.3)	
Other	0	11 (17.2)		14 (21.9)	
Pathologic tumor characteristics, *n* (%)					
Total necrosis among treated patients, *n*/total *n* (%)	1/8 (12.5)	25/43 (58.1)	0.008	7/43 (16.3)	0.741[1]
Within Milan criteria	3 (37.5)	52 (81.3)	0.015	35 (54.7)	0.463[1]
Within UCSF criteria	6 (75.0)	56 (87.5)	0.307	46 (71.9)	> 0.999[1]
Median cumulative nodule size			0.072		0.862[1]
< 2.1	1 (12.5)	21 (32.8)		9 (14.1)	
2.2-5.0	4 (50.0)	37 (57.8)		39 (60.9)	
> 5.1	3 (37.5)	6 (9.4)		16 (25.0)	
Tumor grade, *n*/total *n* (%)			0.225		0.214[1]
1	2 (25.0)	28 (43.8)		4 (6.3)	
2	4 (50.0)	31 (48.4)		40 (62.5)	
3	2 (25.0)	5 (7.8)		20 (31.3)	
Microvascular invasion	3 (37.5)	6 (9.4)	0.056	20 (31.3)	0.741[1]

[1]Fisher exact test.
[2]*t*-test.
[3]Mann-Whitney test. Data expressed as mean ± SD or median (interquartile range). CTP: Child-turcotte-pugh; HBV: Hepatitis B virus; HCV: Hepatitis C virus; LT: Liver transplant; NAFLD: Nonalcoholic fatty liver disease; AFP: Alpha-fetoprotein; TACE: Transarterial chemoembolization; UCSF: University of California San Francisco.



DOI: 10.5306/wjco.v13.i8.688

Figure 1 Flow chart of eligible patients included in the analysis according to the diagnosis of hepatocellular carcinoma, intrahepatic cholangiocarcinoma, or mixed hepatocellular-cholangiocarcinoma on explant pathology (January 1998-July 2019, southern Brazil). LT: Liver transplant; HCC: Hepatocellular carcinoma; HCC-CC: Hepatocellular cholangiocarcinoma; ICC: Intrahepatic cholangiocarcinoma.

characteristics (imaging findings and highest AFP level), whereas the second pairing considered similar explant pathology. There was no statistically significant difference between the two groups (Table 2). Most patients were men, and HCV infection was the most common etiology of liver cirrhosis. Also, there was no statistically significant difference between recurrence, overall mortality, or recurrence-free survival rates in either pairing (by pretransplant or posttransplant tumor characteristics) (Figure 2).

Compared with HCC transplant recipients with similar pretransplant characteristics, patients with HCC-CC showed no significant differences in 1- and 5-year overall mortality (14.3% and 52.4% vs 14.3% and 45.9%; $P = 0.500$) and RFS (85.7% and 47.6% *vs* 85.7% and 54.1%; $P = 0.278$). Compared with those with similar posttransplant characteristics, patients with HCC-CC also showed no statistical differences in 1- and 5-year overall mortality (14.3% and 40.9% *vs* 14.3% and 45;9%; $P = 0.528$) and 1- and 5-year RFS (85.7% and 59.1% vs 85.7% and 54,1%; $P = 0.283$) (Figure 4).

DISCUSSION

The present study described the experience of a Brazilian LT center with the outcomes of LT recipients with ICC or HCC-CC who had a pretransplant radiological diagnosis of HCC. Over a 22-year period, the rate of incorrect diagnosis of ICC or HCC-CC and unintentional LT was 3.1%, similar to that identified in a single-center Spanish study analyzing a 10-year period[24].

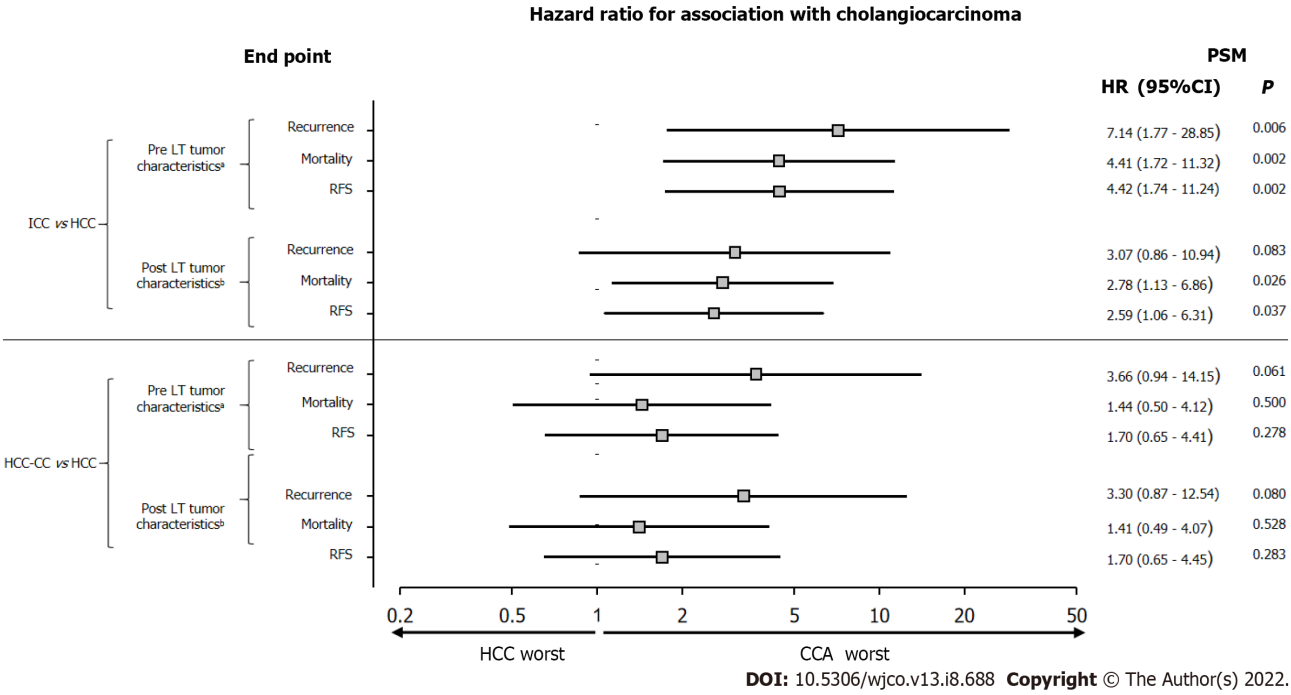
Table 2 Comparison of pretransplant tumor characteristics, locoregional therapy, and posttransplant tumor characteristics between patients with mixed hepatocellular-cholangiocarcinoma and hepatocellular carcinoma, matched 1:8 for pre- liver transplant factors and explant factors

Variable	Pre-LT factors			Explant factors	
	HCC-CC (*n* = 7)	HCC (*n* = 56)	*P* value	HCC (*n* = 56)	*P* value
Recipient characteristics					
Age, mean ± SD	58.0 ± 6.9	60.3 ± 9.2	0.317	60.8 ± 7.1	0.289[2]
Male, *n* (%)	4 (57.1)	41 (73.2)	0.397	4 (57.1)	0.375[1]
Etiology of liver disease, *n* (%)			0.192		0.789[1]
HCV	6 (85.7)	42 (75.0)		41 (73.2)	
Alcohol	0	7 (12.5)		3 (5.4)	
HBV	0	5 (8.9)		5 (8.9)	
NAFLD	1 (14.3)	0		3 (5.4)	
Cryptogenic	0	2 (3.6)		4 (7.1)	
CTP class, *n* (%)			0.201		0.556[1]
A	3 (42.9)	21 (37.5)		29 (51.8)	
B	2 (28.6)	30 (53.6)		20 (35.7)	
C	2 (28.6)	5 (8.9)		7 (12.5)	
Maximum pretransplant AFP, ng/mL	35.3(4.3-357.0)	9.6(1.1-628.0)	0.150	16.5(1.1-6123.0)	0.668[3]
Radiographic tumor characteristics					
Cumulative tumor diameter, cm, *n* (%)			0.224		0.723[t]
< 2.1	1 (14.3)	25 (44.6)		11 (19.6)	
2.2-5.0	4 (57.1)	23 (41.1)		36 (64.3)	
5.1	2 (28.6)	8 (14.3)		9 (16.1)	
Neoadjuvant therapy, *n* (%)			0.085		0.081[1]
None	0	14 (25.0)		12 (21.4)	
TACE	3 (42.9)	22 (39.3)		28 (50.0)	
Other	4 (57.1)	20 (35.7)		16 (28.6)	
Pathologic tumor characteristics, *n* (%)					
Total necrosis among treated patients, *n*/total *n* (%)	3/7 (33.3)	20/42 (47.6)	0.8000	18/44 (40.1)	0.223[1]
Within Milan criteria	4 (57.1)	39 (69.6)	0.669	35 (62.5)	> 0.999[1]
Within UCSF criteria	6 (85.7)	49 (87.5)	> 0.999	45 (80.4)	> 0.999[1]
Median cumulative nodule size			0.224		0.723[1]
< 2.1	1 (14.3)	25 (44.6)		11 (19.6)	
2.2-5.0	4 (57.1)	23 (41.1)		36 (64.3)	
> 5.1	2 (28.6)	8 (14.3)		9 (16.1)	
Tumor grade, *n*/total *n* (%)			0.722		0.233[1]
1	0	1/36 (2.8)		0	
2	4/7 (57.1)	24/36 (66.7)		18/55 (32.7)	
3	3/7 (42.9)	11/36 (30.6)		37/55 (67.3)	
Microvascular invasion	0	9 (16.1)	0.580	0	0

[1]Fisher exact test.

[2]*t*-test.

[3]Mann-Whitney test. Data expressed as mean ± SD or median (interquartile range). CTP: Child-turcotte-pugh; HBV: Hepatitis B virus; HCV: Hepatitis C virus; LT: Liver transplant; NAFLD: Nonalcoholic fatty liver disease; AFP: Alpha-fetoprotein; TACE: Transarterial chemoembolization; UCSF: University of California San Francisco.



DOI: 10.5306/wjco.v13.i8.688

Figure 2 Post-liver transplant 3-year risk of recurrence, overall mortality, and recurrence-free survival in patients with intrahepatic cholangiocarcinoma or mixed hepatocellular-cholangiocarcinoma compared with patients with hepatocellular carcinoma, matched 1:8 for pretransplant tumor characteristics (Pre liver transplant characteristics) and pathologic tumor characteristics (Post liver transplant characteristics). [a]Pre liver transplant (LT) characteristics: serum alpha-fetoprotein and radiologic tumor diameter; [b]Post LT characteristics: Tumor diameter, grade/differentiation, microvascular invasion. PSM: Propensity score matching; LT: Liver transplant; HCC: Hepatocellular carcinoma; HCC-CC: Hepatocellular cholangiocarcinoma; ICC: Intrahepatic cholangiocarcinoma.

In order to assess outcomes of these entities (ICC or HCC-CC) after LT, we compared the outcomes of patients who had ICC or HCC-CC with the outcomes of patients transplanted for HCC. At first, we matched LT recipients with ICC and LT recipients with HCC for pretransplant tumor characteristics. Patients with ICC were more likely to have poorer tumor differentiation and higher microvascular invasion rates on explant pathology. To estimate the risk of recurrence, overall mortality, and recurrence-free survival in both groups, we used PSM followed by simple Cox regression. This comparative, propensity-matched analysis showed a higher risk of poorer outcomes after LT for ICC than HCC when patients were matched for pretransplant tumor characteristics. A previous study reported that worse tumor differentiation and presence of microvascular invasion are risk factors for recurrence in LT recipients with ICC[25]. Therefore, in order to assess the role of the potentially more aggressive nature of ICC, we matched patients with ICC and patients with HCC for explant pathology, which included nuclear differentiation, microvascular invasion, and cumulative tumor diameter, and repeated the same statistical analyses. Again, patients with ICC had worse outcomes (tumor recurrence, overall mortality, and recurrence-free survival) than those with HCC. That is, ICC was associated with worse outcomes even when high-risk factors for tumor recurrence were considered, indicating that ICC is an inherently more aggressive tumor whose risk factors for recurrence differ from those traditionally described for HCC. To our knowledge, this is the first time that posttransplant outcomes of patients with ICC and HCC have been comparatively evaluated by matching patients for explant pathology.

LT has been contraindicated in patients with ICC due to poor results[26-28]. The possibility of successfully transplanting patients with ICC began to change as it became clear that better patient selection was likely to impact posttransplant outcomes. Satisfactory results have been recently reported in LT of cirrhotic patients with grafts showing incidental ICC on explant pathology. Retrospective data from these patients demonstrated suitable 5-year overall and recurrence-free survival in patients with "very early" ICC (≤ 2 cm)[18,25,29]. A Japanese study found that patients with and without cirrhosis who underwent liver resection for ICC ≤ 2 cm reached a 100% 5-year survival rate. The authors identified 2 cm as a good cutoff point when selecting patients for hepatectomy[30]. Recently, French

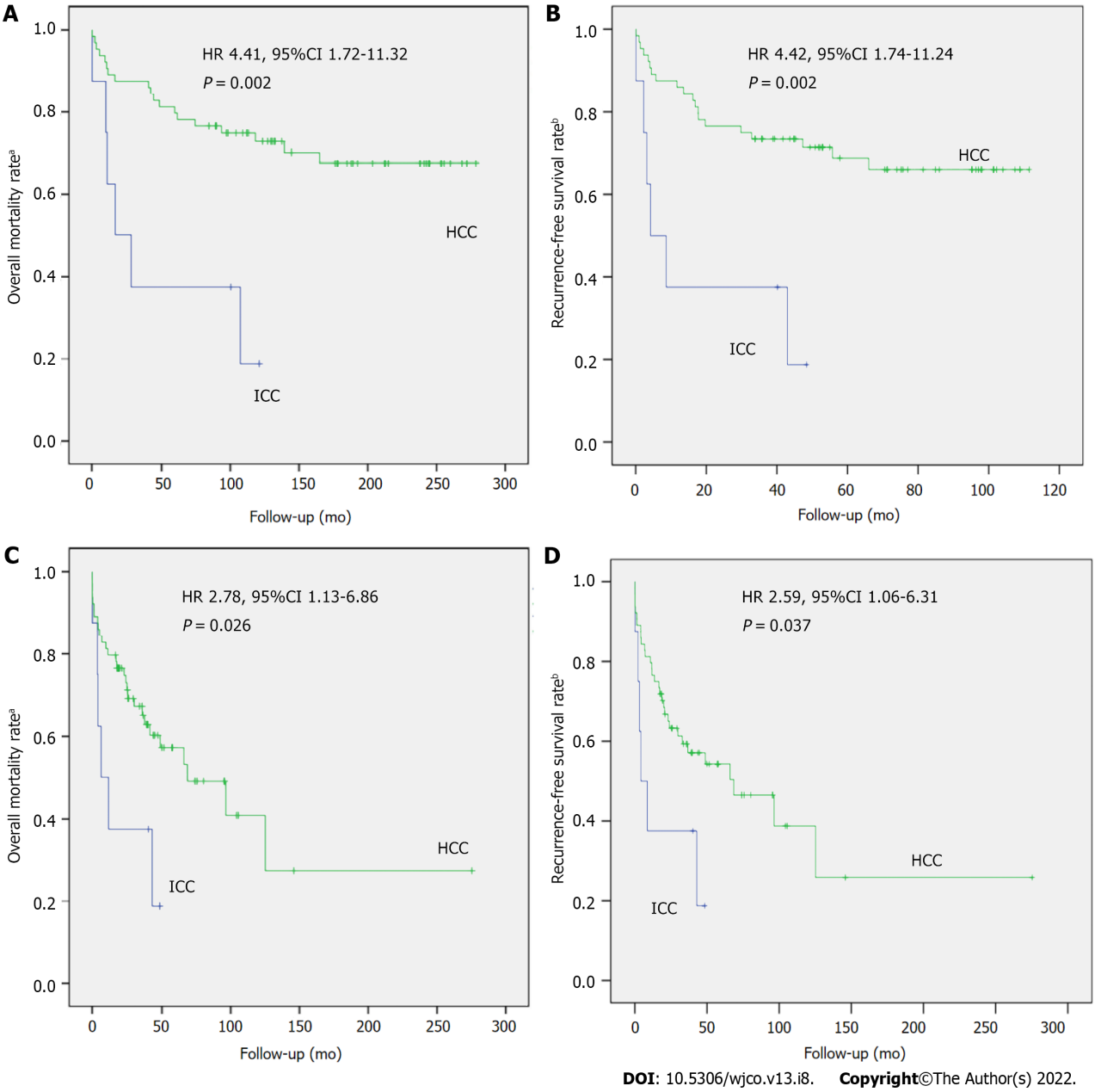


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Figure 3 Kaplan-Meier curves representing. A: post-liver transplant overall survival; B: recurrence-free survival in patients with intrahepatic cholangiocarcinoma compared with patients with hepatocellular carcinoma, matched 1:8 for pretransplant tumor characteristics; C: post-liver transplant overall survival; D: recurrence-free survival in patients with intrahepatic cholangiocarcinoma compared with patients with hepatocellular carcinoma matched 1:8 for posttransplant tumor characteristics. CIs: Confidence intervals; HCC: hepatocellular carcinoma; HRs: Hazard ratios; ICC: intrahepatic cholangiocarcinoma.

researchers suggested that this ≤ 2 cm limit could be expanded by showing, in a retrospective multicenter study analyzing posttransplant outcomes of cirrhotic patients with incidental ICC detected on the pathological examination of the explant, that patients with clearly differentiated ICC up to 3 cm had similar survival to patients with tumors ≤ 2 cm. In this study, the only independent variable associated with tumor recurrence was its differentiation[31]. Prospective multicenter clinical trials are needed to confirm these results. The 2 cm cutoff point seems safe but limited because preoperative radiological diagnosis of these small tumors is challenging[15,16] and ICC features are still often underestimated during pre-LT diagnostic evaluation. Nevertheless, studies indirectly state that ICC is a more aggressive tumor by suggesting that LT should only be an option for patients with tumors ≤ 2 cm. This differs from the indication for LT in patients with HCC, who can undergo LT with tumors up to 5 cm in diameter, with acceptable recurrence rates[32]. It is important to note that, in our series, all patients with liver cirrhosis had ICCs > 2 cm. In order to expand the indication criteria for LT in patients with liver cirrhosis and unresectable ICC, the effectiveness of pretransplant neoadjuvant chemotherapy is being evaluated[33]. The International Liver Transplantation Society (ILTS) recommends resection as the treatment of choice for patients with ICC. When the procedure is contraindicated, LT may be considered when the tumor is ≤ 2 cm; if the tumor is > 2 cm, LT may be performed under strict clinical

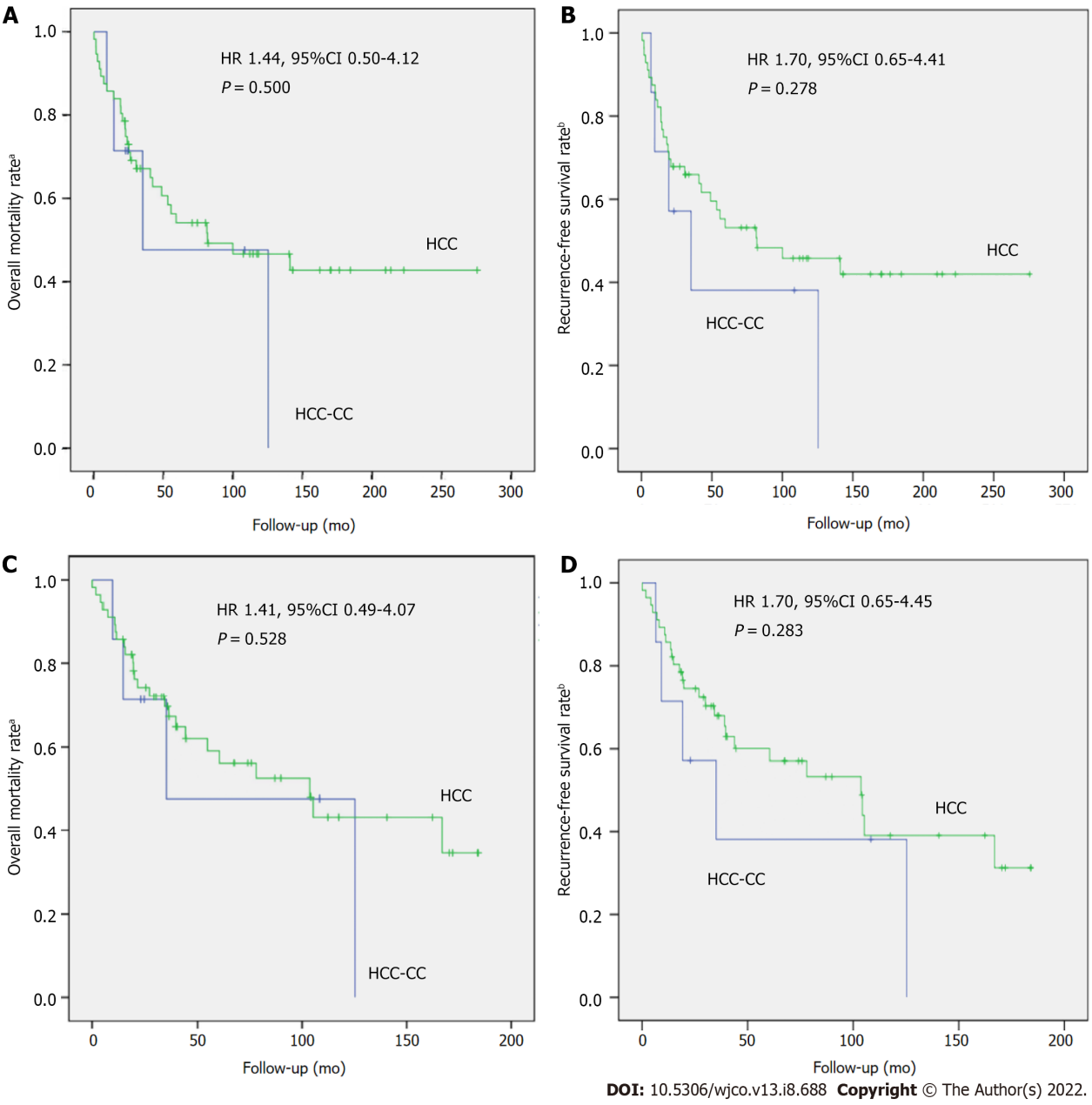

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Figure 4 Kaplan-Meier curves representing. A: post-liver transplant overall survival; B: recurrence-free survival in patients with mixed hepatocellular-cholangiocarcinoma compared with patients with hepatocellular carcinoma (HCC), matched 1:8 for pretransplant tumor characteristics; C: post-liver transplant overall survival; D: recurrence-free survival in patients with mixed hepatocellular-cholangiocarcinoma compared with patients with hepatocellular carcinoma matched 1:8 for posttransplant tumor characteristics. HCC: hepatocellular carcinoma; HCC-CC: hepatocellular-cholangiocarcinoma; HRs: Hazard ratios; CIs: Confidence intervals.

protocols and only when the disease remains stable after neoadjuvant therapy[34].

We performed the same comparisons, using pretransplant and posttransplant tumor characteristics, for LT recipients with HCC-CC *vs* HCC, but no statistically significant differences were observed between the two groups. The statistical analyses (PSM and simple Cox regression) yielded similar risks for tumor recurrence, overall mortality, and recurrence-free survival when patients were matched for pretransplant or posttransplant tumor characteristics. As observed in ICC, patients with HCC-CC also had a worse prognosis than those with HCC, but the differences were smaller than those found for ICC *vs* HCC; consequently, in most outcomes, the differences did not reach statistical significance. This may suggest that LT recipients with ICC or HCC-CC have worse outcomes than those with HCC, but ICC appears to be more aggressive. Lunsford *et al*[35] analyzed posttransplant outcomes of 12 patients with HCC-CC *vs* 36 patients with HCC matched for the pretransplant and posttransplant variables reproduced in the present study. When patients were matched for explant pathology, those with HCC-CC had a slightly higher recurrence rate, without statistical significance, whereas recurrence-free survival and overall survival rates were equivalent to those of LT recipients with HCC[35]. Other authors also consider that a diagnosis of HCC-CC should not be an impediment to LT in well-selected cases[24,36,37]. However, for patients with HCC-CC, the ILTS expert panel believes that this tumor is

not an established indication for LT due to the limited worldwide experience, and prognostic factors need to be identified to improve patient selection and to obtain better results with the procedure[30].

Transplant oncology is a new concept encompassing multiple disciplines of transplantation medicine and oncology (transplant oncologists, hepatologists, gastroenterologists, transplant hepatobiliary surgeons, interventional radiologists, and immunologists) designed to push the envelope of the treatment and research of hepatobiliary cancers[38,39]. This field will certainly improve treatments and cure rates for patients with HCC, ICC, or HCC-CC, as well as other cancer types.

This study has limitations that need to be addressed. First, it is a retrospective study conducted at a single center with a limited number of cases. However, given the rarity of these tumors, most studies are retrospective and have also included a small number of patients, which makes it difficult to perform statistical analyses that can identify factors potentially associated with the outcomes[40]. Furthermore, because LT is a current contraindication for patients with ICC or HCC-CC, the diagnosis was made on explant. Finally, the study included patients receiving care over a long period of time. To minimize any bias that may have resulted from advances in research, management, and treatment during the study period, patients were also matched for year of transplant.

CONCLUSION

In this series, LT for ICC (all excepted one were larger than 2 cm) was associated with worse outcomes compared with LT for HCC, even when patients were matched for explant pathology. However, the outcomes after LT for mixed HCC-CC, despite being worse than those of LT recipients with HCC, did not reach statistical significance. Improvement in the detection of these rare tumors during pretransplant evaluation is essential for the eventual adoption of LT as an effective treatment for these patients.

ARTICLE HIGHLIGHTS

Research background

Cholangiocarcinoma (CC) is a rare tumor that arises from the epithelium of the bile ducts. It is classified according to anatomic location as intrahepatic, perihilar, or distal. Intrahepatic cholangiocarcinoma (ICC) is rare in patients with cirrhosis due to causes other than primary sclerosing cholangitis. Mixed hepatocellular-cholangiocarcinoma (HCC-CC) is a rare neoplasm with histologic findings of both hepatocellular carcinoma (HCC) and ICC within the same tumor mass.

Research motivation

Because of difficulties in reaching the correct diagnosis, patients eventually undergo liver transplantation (LT) with a presumptive diagnosis of HCC on imaging when, in fact, they have ICC or HCC-CC.

Research objectives

To determine the prevalence of ICC or HCC-CC confirmed by explant pathology in patients who underwent LT with the presumptive diagnosis of HCC and to compare tumor recurrence (TR), recurrence-free survival (RFS), and overall mortality (OM) rates between these patients and LT recipients with HCC.

Research methods

This retrospective cohort study included patients aged ≥ 18 years with liver cirrhosis and imaging findings suggestive of HCC within the Milan criteria who underwent LT between June 1997 and July 2019. Patients were divided into three groups according to the diagnosis on explant pathology: (1) Patients with HCC; (2) Patients with ICC; and (3) Patients with mixed HCC-CC. The analyzed outcomes were TR, RFS, and OM. Propensity score matching was used to assess whether TR, OM, and RFS rates in patients with ICC or HCC-CC differed from those in patients with HCC. Additionally, hazard ratios (HRs) and their confidence intervals were calculated. Progression-free survival and OM rates were computed with the Kaplan-Meier method using Cox regression for comparison.

Research results

Over a 22-year period, 475 patients with the presumptive diagnosis of HCC underwent LT, and 15 (3.1%) were found to have either ICC (n = 8) or HCC-CC (n = 7) detected in the pathological examination of the explant. LT recipients with ICC had higher TR (46% *vs* 11%; P = 0.006), higher OM (63% *vs* 23%; P = 0.002), and lower RFS (38% *vs* 89%; P = 0.002) than those with HCC when matched for pretransplant tumor characteristics, as well as higher TR (46% *vs* 23%; P = 0.083), higher OM (63% *vs*

35%; $P = 0.026$), and lower RFS (38% *vs* 59%; $P = 0.037$) when matched for posttransplant tumor characteristics. Two pairings were performed to compare the outcomes of LT recipients with HCC-CC *vs* HCC. There was no significant difference between the outcomes in either pairing.

Research conclusions

Patients with ICC had worse outcomes than patients with HCC undergoing LT. Preoperative diagnosis of HCC-CC should not prompt the exclusion of these patients from transplant options.

Research perspectives

This study reinforces the need for more accurate criteria: (1) To identify these rare tumors in pretransplant evaluation; and (2) To select patients who may benefit from LT.

ACKNOWLEDGEMENTS

To the Liver Transplantation Group at Santa Casa de Misericórdia de Porto Alegre, RS, Brazil (Guido Cantisani Team), and to the Hospital Vozandes Quito-HVQ AS, Quito, Ecuador.

FOOTNOTES

Author contributions: All the authors solely contributed to this paper.

Institutional review board statement: The Institutional Review Board of Santa Casa de Misericórdia de Porto Alegre approved the study protocol (No. 4.250.889).

Informed consent statement: Informed consent was waived due to the non-interventional design of the study and retrospective nature of data collection. All investigators signed a data use agreement to ensure the ethical and secure use of the data.

Conflict-of-interest statement: All authors have no conflicts of interest to disclose.

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE Statement checklist of items, and the manuscript was prepared and revised according to the STROBE Statement checklist of items.

Country/Territory of origin: Brazil

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S-Editor: Wang LL
L-Editor: A
P-Editor: Qi WW

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World Journal of Clinical Oncology

Submit a Manuscript: https://www.f6publishing.com

World J Clin Oncol 2022 August 24; 13(8): 702-711

DOI: 10.5306/wjco.v13.i8.702

ISSN 2218-4333 (online)

ORIGINAL ARTICLE

Retrospective Study

Computer-aided clinical image analysis as a predictor of sentinel lymph node positivity in cutaneous melanoma

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Specialty type: Oncology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

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

P-Reviewer: Mangla A, United States; Wang P, China

Received: February 28, 2022
Peer-review started: February 28, 2022
First decision: May 31, 2022
Revised: June 24, 2022
Accepted: July 26, 2022
Article in press: July 26, 2022
Published online: August 24, 2022

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Abstract

BACKGROUND

Delays in sentinel lymph node (SLN) biopsy may affect the positivity of non-SLNs. For these reasons, effort is being directed at obtaining reliable information regarding SLN positivity prior to surgical excision. However, the existing tools, *e.g.*, dermoscopy, do not recognize statistically significant predictive criteria for SLN positivity in melanomas.

AIM

To investigate the possible association of computer-assisted objectively obtained color, color texture, sharpness and geometry variables with SLN positivity.

METHODS

We retrospectively reviewed and analyzed the computerized medical records of all patients diagnosed with cutaneous melanoma in a tertiary hospital in Germany

during a 3-year period. The study included patients with histologically confirmed melanomas with Breslow > 0.75 mm who underwent lesion excision and SLN biopsy during the study period and who had clinical images shot with a digital camera and a handheld ruler aligned beside the lesion.

RESULTS

Ninety-nine patients with an equal number of lesions met the inclusion criteria and were included in the analysis. Overall mean (± standard deviation) age was 66 (15) years. The study group consisted of 20 patients with tumor-positive SLN (SLN+) biopsy, who were compared to 79 patients with tumor-negative SLN biopsy specimen (control group). The two groups differed significantly in terms of age (61 years *vs* 68 years) and histological subtype, with the SLN+ patients being younger and presenting more often with nodular or secondary nodular tumors ($P < 0.05$). The study group patients showed significantly higher eccentricity (*i.e.* distance between color and geometrical midpoint) as well as higher sharpness (*i.e.* these lesions were more discrete from the surrounding normal skin, $P < 0.05$). Regarding color variables, SLN+ patients demonstrated higher range in all four color intensities (gray, red, green, blue) and significantly higher skewness in three color intensities (gray, red, blue), $P < 0.05$. Color texture variables, *i.e.* lacunarity, were comparable in both groups.

CONCLUSION

SLN+ patients demonstrated significantly higher eccentricity, higher sharpness, higher range in all four color intensities (gray, red, green, blue) and significantly higher skewness in three color intensities (gray, red, blue). Further prospective studies are needed to better understand the effectiveness of clinical image processing in SLN+ melanoma patients.

Key Words: Melanoma; Skin cancer; Image processing; Sentinel lymph node; Presurgical

Core Tip: Computer-aided image analysis can facilitate prediction of sentinel lymph-node positivity. Several color, sharpness and geometry parameters can predict positive lymph node occurrence, while color texture cannot determine sentinel lymph node positivity.

Citation: Papadakis M, Paschos A, Papazoglou AS, Manios A, Zirngibl H, Manios G, Koumaki D. Computer-aided clinical image analysis as a predictor of sentinel lymph node positivity in cutaneous melanoma. *World J Clin Oncol* 2022; 13(8): 702-711

URL: https://www.wjgnet.com/2218-4333/full/v13/i8/702.htm

DOI: https://dx.doi.org/10.5306/wjco.v13.i8.702

INTRODUCTION

Cutaneous melanoma is a highly aggressive tumor that often spreads to local lymph nodes. Sentinel lymph node biopsy (SLNB) is commonly performed to identify nodal metastases because sentinel lymph node (SLN) status is a strong prognostic factor for survival in melanoma patients, especially in those without evidence of clinically positive lymph nodes[1]. In some subgroups, *e.g.*, in patients with thick melanomas (*i.e.* Breslow thickness > 4 mm) and patients with melanomas of the scalp, SLN status is considered the most important prognostic survival factor[2,3].

According to the existing guidelines, the decision for SLNB is based on the thickness of the primary tumor. SLNB is indicated for all primary tumors thicker than 1 mm and tumors thicker than 0.75 mm in the presence of ulceration or high mitotic rate (> 1 mm²). SLNB is of crucial importance in disease management because positive SLNB should be followed by lymph node dissection for regional disease control and staging purposes. Delays in SLNB may affect the positivity of non-SLNs[4]. For these reasons, an effort is being directed at obtaining reliable information regarding SLN positivity before surgical excision.

Dermoscopy is a non-invasive technique that facilitates early melanoma detection by revealing skin features invisible to the naked eye. However, dermoscopy does not recognize statistically significant predictive criteria for SLN positivity in melanomas because specific melanoma criteria strongly associated with a higher Breslow thickness, such as gray-blue areas or an atypical vascular pattern, do not seem to associate with SLN positivity[5].

Computer-aided clinical image analysis is also used to improve diagnostic accuracy for skin melanoma. We have shown that geometrical and color parameters objectively extracted by computer-aided clinical image processing may correlate with tumor thickness in patients with cutaneous melanoma[6]. However, to the best of our knowledge, there is no study investigating the possible association of computer-assisted objectively obtained color, color texture, sharpness and geometry variables with SLN positivity. The aim of this study was to investigate whether such an association exists.

MATERIALS AND METHODS

Patient recruitment

We retrospectively reviewed and analyzed the computerized medical records of all patients diagnosed with cutaneous melanoma in a tertiary hospital in Germany during a 3-year period. The study included patients with histologically confirmed melanomas with Breslow > 0.75 mm who underwent lesion excision and SLN biopsy during the study period and who had clinical images shot with a digital camera and a handheld ruler aligned beside the lesion. Patients with melanomas with Breslow < 0.75 mm and in situ melanomas as well as patients without digital images were excluded from the study. Patients referred to our center after primary excision to undergo SLN biopsy were also excluded from the study.

The study group consisted of patients with a positive SLN biopsy who were compared to patients with a negative SLN biopsy (control group). Clinical features studied included age, sex, tumor location and diagnosis date. Histopathologic features included tumor subtype [superficial spreading (de novo and nevus-associated) and nodular, including secondary nodular], Breslow thickness, Clark level, presence of ulceration, nevus pre-existence and SLN status (positive or negative). The study was approved by the institutional review board of the University of Witten-Herdecke.

Image collection, storage system and image database

All lesions were photographed at admission with the same commercial digital camera at a resolution of 1600 × 1200 pixels and with a handheld ruler aligned beside the lesion to allow for correct image scaling. All photos were obtained from the same educated nurse to minimize inconsistencies in methodology and were uploaded to a local server. The lesions were then excised under local anesthesia, and the diagnosis was histologically confirmed.

Image processing

The color images obtained underwent digital processing with an almost fully automated noncommercial software developed by one of the authors for study purposes. The software applies several kinds of algorithms to allow image segmentation and geometry, color and color texture analysis (Figure 1). The only manual involvement was the selection of the lesion border with the mouse cursor when the algorithm failed to do so (*i.e.* in very small lesions) (Figure 2). Such cases were independently analyzed twice by two of the authors (MP and GM) to avoid intraobserver errors (Figure 3). In cases of discrepancies, the mean scores were accepted and further analyzed. The 34 variables studied are classified as follows: (1) Geometrical variables (*i.e.* area, maximum diameter, perimeter, circularity, eccentricity and mean radius); (2) Color variables [*i.e.* range, standard deviation, coefficient of variation and skewness for all four color intensities (gray, red, green, blue)]; (3) Sharpness variables; and (4) Color texture variables (*i.e.* lacunarity)[7]. All variables are thoroughly described in Table 1.

Statistical analysis

Normal distribution was determined using histogram plots, box plots and the Shapiro-Wilk test. Continuous data are presented in mean-standard deviation form. Categorical variables were compared using the two-tailed Fisher's exact test and continuous variables using the two-tailed Student's *t* test. A *P* value of less than 0.05 was considered statistically significant. Univariate and multivariate analysis using logistic regression were employed to identify potential independent determinants of a positive SLN result. Data analyses were performed using SPSS 23.

RESULTS

Ninety-nine patients with an equal number of lesions met the inclusion criteria and were included in the analysis. Of these, 52 (52%) were males and the rest (48%) females. Histogram plots, box plots and the Shapiro-Wilk test demonstrated an almost normal distribution appearance for all continuous variables. The overall mean (standard deviation) age was 66 (15) years. The youngest patient was 14-years-old and the oldest 92-years-old. The study group consisted of 20 patients with tumor-positive SLN (SLN+) biopsy who were compared to 79 patients with tumor-negative SLN biopsy specimens (control group).

Table 1 Explanation of geometric, sharpness, color and color texture variables studied[7]

Classification	Parameter	Explanation
Geometry variables	Area	Lesion surface area, measured in cm^2
	Maximum diameter	The longest line that joins two points on the border of the lesion, measured in cm
	Perimeter	Total boundary length of the region of interest (*i.e.* lesion), measured in cm
	Circularity	Ratio of the perimeter of the lesion divided by the perimeter of a circle with the same midpoint and same area as the lesion
	Mean radius (Rm)	Mean value of the lesion's radii
	Standard deviation of Rm	Standard deviation of the mean radius
	Coefficient of variation of Rm	Expresses the standard deviation as a percentage of the mean
	Eccentricity	Distance between color and geometric midpoint within the lesion
	Eccentricity ratio	Distance between midpoint and color midpoint expressed as a fraction of the maximum diameter
Sharpness variables	SD of gray intensity	Intensity of gray on the border of the lesion
	Coefficient of variation of SD of gray intensity	SD of gray intensity in grayscale image. The higher the value, the more discrete the lesion is from the surrounding normal skin (Manousaki *et al*[7], 2016)
Color texture variables	Grayscale lacunarity of lesion (Lac gray)	It is estimated in grayscale image and assesses image texture heterogeneity or incomplete space filling within the lesion
Color variables	Range of gray, red, green, blue	Range of values of gray, red, green, blue intensity
	Mean gray, red, green, blue	Mean value of gray, red, green, blue intensity within the lesion
	SD of gray, red, green, blue	Standard deviation of gray, red, green, blue intensity within the lesion
	Coefficient of variation of gray, red, green, blue	Expresses the standard deviation of gray, red, green, blue intensity values as mean percentage
	Skewness from Gaussian curve (gray, red, green, blue)	Deviation of each color's histogram from the normal distribution curve

SD: Standard deviation.

The two groups differed significantly in terms of age (61 years *vs* 68 years) and histological subtype; the SLN+ patients were younger and presented more often with nodular or secondary nodular tumors ($P < 0.05$).

The study group patients also showed significantly higher eccentricity (*i.e.* the distance between color and geometrical midpoint) and higher sharpness (*i.e.* these lesions were more discrete from the surrounding normal skin, $P < 0.05$). Regarding color variables, SLN+ patients demonstrated a higher range in all four color intensities (gray, red, green, blue) and significantly higher skewness in three color intensities (gray, red, blue), $P < 0.05$. Color texture variables (*i.e.* lacunarity) were similar in both groups. Comparative data are summarized in Table 2.

Multivariate analysis of univariately significant variables ($P < 0.05$) revealed that younger age and higher eccentricity were independently associated with a higher probability of positive lymph node occurrence [for age: adjusted odds ratio (aOR) = 0.95, 95% confidence interval (CI): 0.91 to 0.99 and for eccentricity: aOR = 1.45, 95%CI: 1.12 to 1.89]. Nevus, nodular and secondary nodular histotypes were also significantly linked with higher odds of positive lymph node presence when compared to the superficial spreading histotype (for nevus: aOR = 14.19, 95%CI: 1.15 to 174.76, for nodular: aOR = 10.71, 95%CI: 1.48 to 77.48 and for secondary nodular: aOR = 18.21, 95%CI: 2.19 to 151.22). The proposed multivariate model can predict the presence of SLN+ with an accuracy of 85% and is summarized in Table 3.

DISCUSSION

Computer-aided image analysis is a noninvasive method and as such an established tool in the physicians' armamentarium to obtain reliable information regarding malignancy before surgical excision. SLN status is a strong prognostic factor for survival in melanoma patients (the tumor thickness threshold for SLNB being 1 mm) in the absence of risk factors. We herein investigated the possible association of computer-assisted objectively obtained color, texture and geometric variables with SLN

Table 2 Demographic, clinical and image processing characteristics of melanoma patients with positive and negative sentinel lymph node biopsy

Variable		Study group, SLNB+; *n* = 20	Control group, SLNB-; *n* = 79	*P* value
Demographics				
Sex				
Male		12 (60%)	40 (50%)	0.62
Female		8 (40%)	39 (50%)	
Age (yr)		61 (13)	68 (15)	0.05
Tumor thickness (mm)		2.6 (2.7)	2.2 (3.0)	0.64
Subtype	Superficial spread	3 (15%)	44 (56%)	0.04
	Nodular	10 (50%)	25 (32%)	
	Secondary nodular	7 (35%)	10 (12%)	
Geometric variables				
Area (cm^2)		3.4 (2.9)	2.8 (4.7)	0.49
MaxD (cm)		2.4 (1.2)	2 (1.1)	0.22
Perimeter (cm)		6.6 (3.0)	5.7 (3.3)	0.25
Circularity (ratio)		1.1 (0.1)	1.1 (0.1)	0.44
Rm (cm)		0.98 (0.5)	0.84 (0.5)	0.21
SDRm		0.14 (0.1)	0.12 (0.1)	0.48
CVRm		13 (7.1)	13 (5.5)	0.88
Delta (cm)		0.04 (0.03)	0.03 (0.03)	0.04
Delta ratio		1.8 (0.9)	1.6 (1.3)	0.42
Sharpness variables				
Sharpness		31 (9)	26 (8)	0.02
CV sharpness		22 (6)	18 (7)	0.02
Color texture variables				
Lac gray		2 (0.28)	1.97 (0.30)	0.65
Color variables				
Mean gray		104 (21)	113 (24)	0.12
SD gray		32 (6)	30 (7)	0.12
CV gray		8 (2)	10 (1)	0.07
Range gray		201 (21)	187 (32)	0.03
Skewness gray		0.52 (0.5)	0.23 (0.6)	0.03
Mean red		144 (26)	157 (32)	0.06
SD red		37 (9)	33 (11)	0.14
CV red		27 (10)	23 (11)	0.14
Range red		205 (23)	190 (37)	0.04
Skewness red		0.03 (0.43)	-0.04 (0.83)	0.003
Mean green		89 (23)	96 (23)	0.24
SD green		33 (7)	31 (6)	0.19
CV green		39 (12)	35 (13)	0.13
Range green		209 (24)	195 (33)	0.04

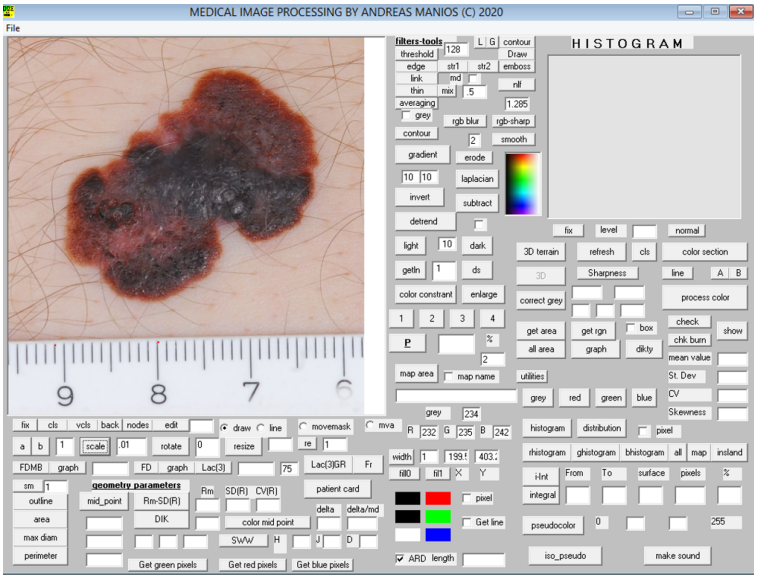
Skewness green	0.64 (0.46)	0.41 (0.60)	0.08
Mean blue	87 (22)	94 (25)	0.22
SD blue	33 (7)	31 (6)	0.24
CV blue	40 (10)	35 (13)	0.14
Range blue	218 (23)	201 (34)	0.02
Skewness blue	0.67 (0.47)	0.42 (0.56)	0.05

All continuous variables were normally distributed and therefore expressed in mean-deviation form. Categorical variables were expressed in terms of absolute (n) and relative (%) frequencies. Statistical significance: *P* value < 0.05. SD: Standard deviation; CV: Coefficient of variation; MaxD: Maximum diameter; SLNB+: Tumor-positive sentinel lymph node biopsy; SLNB-: Tumor-negative sentinel lymph node biopsy; Rm: Mean radius; SDRm: Standard deviation of the mean radius; CVRm: Coefficient variation of the mean radius; Lac: Lacunarity.

Table 3 Multivariate analysis of univariately significant predictors of a positive sentinel lymph node result

Variable	Coefficient (β)	Standard Error	Wald χ^2	*P* value	Odds ratio	95%CI
Age	-0.05	0.20	7.60	0.006	0.95	0.91 to 0.99
Subtype, nevus-associated	2.65	1.28	4.28	0.038	14.19	1.15 to 174.76
Subtype, nodular	2.37	1.01	5.51	0.019	10.71	1.48 to 77.48
Subtype, secondary nodular	2.90	1.08	7.22	0.007	18.21	2.19 to 151.22
Eccentricity	0.38	0.13	7.84	0.005	1.46	1.12 to 1.89

95%CI: The 95% confidence interval for the estimated odds ratios.



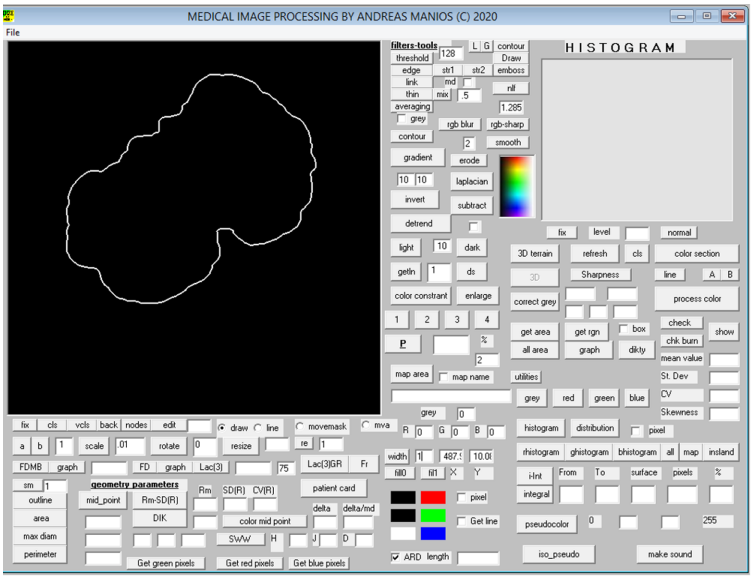
DOI: 10.5306/wjco.v13.i8.702

Figure 1 Scaling of the lesion.

positivity.

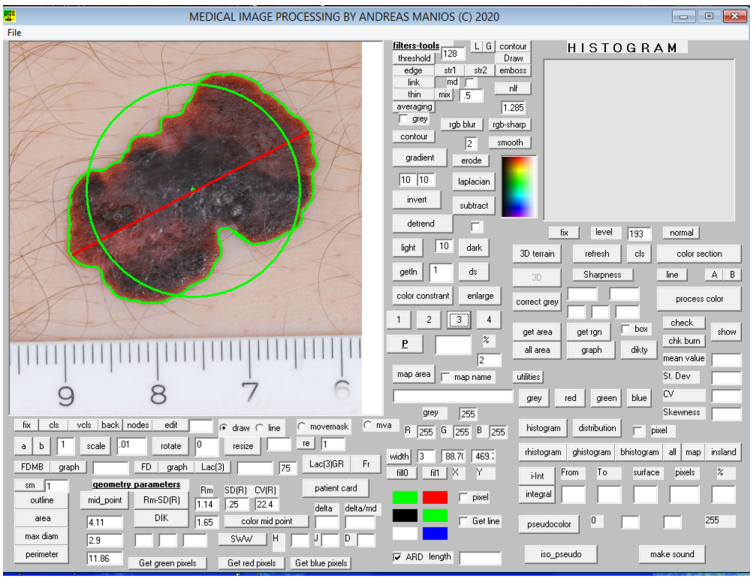
SLN+ patients have a higher range in all four color intensities (gray, red, green, blue) and significantly higher skewness in three color intensities (gray, red, blue). Blue and black pigmentation is associated with the presence of nodular melanoma, which in our study accounted for 50% of the SLN+ tumors[8]. Malignant epidermal structures, (*e.g.*, atypical pigment network, radial streaks and pseudopods) are rarely seen in SLN+ melanomas, while they are observed in one-fourth of SLN negative lesions[5].

Despite the significant differences in all color intensities, we found that lacunarity (a measure of the variation of the color intensity) cannot predict SLN status in melanoma patients although it is a proven



DOI: 10.5306/wjco.v13.i8.702

Figure 2 Automatic recognition of the lesion's borders.



DOI: 10.5306/wjco.v13.i8.702

Figure 3 Measurements.

promising parameter in the automated differentiation of melanoma from non-melanoma[9]. We also found the lesions of SLN+ patients have significantly higher eccentricity, which is an index of uneven lesion coloration. Eccentricity represents a special case of asymmetry, and as shown before eccentric lesions may be thicker[6]. Dermoscopically, only the presence of ulceration and blotch correlate with positive SLNB[10].

Regarding histological type, González-Álvarez *et al*[10] found nodular melanomas to be the most associated with SLN positivity, reporting an OR of 3.98. This is consistent with our findings, where half of the SLN+ tumors were nodular, and the OR was 14.4. Moreover, we found that secondary nodular tumors are much more often associated with SLN+, with the OR exceeding 25. This may reflect more aggressive tumor growth because angiolymphatic invasion is observed in the majority of nodular melanomas with SLN+[5]. According to our findings, a multivariate model consisting of age, histological type and eccentricity can predict the presence of SLN+ with an accuracy of up to 85%.

A recent study described a deep learning-based digital biomarker to predict SLN+ from digitized hematoxylin and eosin slides of primary melanoma tumors. Artificial neural networks predicted SLN status with an accuracy of 55%-62%[11]. This relatively low accuracy is attributed to morphological changes of the tumor cells or tumor architecture. Moreover, the histopathological workup may have

caused tumor cells to be missed in the lymph nodes. Neural networks failed to detect features other than thickness and age that predict SLN+. We found that higher values of eccentricity, sharpness, blue, gray, green range, red skewness and red mean could also predict positive lymph node occurrence.

Our study is limited regarding its retrospective nature and small sample size. First, it is a single-center study of German individuals, and therefore the results cannot be easily generalized. Second, only melanoma patients with available clinical images made before the diagnosis was established were included. Therefore, there is a high risk of bias due to no consecutive cases being included. Selection bias may have led to suspect cases being more frequently photographed. Moreover, we excluded all patients with melanomas with Breslow thickness < 0.75 mm. However, the specimens were analyzed by different pathologists during the study period, so the interpretation bias of tumor thickness may have led to eligible cases being excluded.

CONCLUSION

In conclusion, computer-aided image analysis can facilitate the prediction of SLN+. SLN+ patients demonstrated significantly higher eccentricity, higher sharpness and higher range in all four color intensities (gray, red, green, blue) as well as significantly higher skewness in three color intensities (gray, red, blue). Further prospective studies are needed to better understand the effectiveness of clinical image processing in SLN+ melanoma patients.

ARTICLE HIGHLIGHTS

Research background

Computer-aided clinical image analysis is used to improve diagnostic accuracy for skin melanoma.

Research motivation

To the best of our knowledge, there is no study investigating the possible association of computer-assisted objectively obtained color, color texture, sharpness and geometry variables with sentinel lymph node positivity (SLN+).

Research objectives

To investigate a possible association of computer-assisted objectively obtained color, color texture, sharpness and geometry variables with SLN+.

Research methods

The study included patients with histologically confirmed melanomas with Breslow > 0.75 mm who underwent lesion excision and SLN biopsy during the 3-year study period and who had clinical images shot with a digital camera and a handheld ruler aligned beside the lesion. All the color images obtained underwent digital processing with an almost fully automated noncommercial software developed by one of the authors for study purposes.

Research results

Ninety-nine patients with an equal number of lesions met the inclusion criteria and were included in the analysis. The study group consisted of 20 patients with SLN+ biopsy who were compared to 79 patients with tumor-negative SLN biopsy specimen (control group). The study group patients showed significantly higher eccentricity (*i.e.* distance between color and geometrical midpoint) as well as higher sharpness (*i.e.* these lesions were more discrete from the surrounding normal skin, $P < 0.05$). Regarding color variables, SLN+ patients demonstrated higher range in all four color intensities (gray, red, green, blue) and significantly higher skewness in three color intensities (gray, red, blue), $P < 0.05$. Color texture variables, *i.e.* lacunarity, were comparable in both groups.

Research conclusions

Computer-aided image analysis can facilitate the prediction of SLN+. SLN+ patients demonstrated significantly higher eccentricity, higher sharpness and higher range in all four color intensities (gray, red, green, blue) as well as significantly higher skewness in three color intensities (gray, red, blue).

Research perspectives

Further prospective studies are needed to better understand the effectiveness of clinical image processing in SLN+ melanoma patients.

FOOTNOTES

Author contributions: Papadakis M designed the research, performed the research, analyzed the data and wrote the paper; Paschos A participated in the data collection; Papazoglou A analyzed the data, Manios A contributed a new software used for the study; Zirngibl H contributed literature sources; Manios G analyzed the data; Koumaki D designed the research, performed the research, analyzed the data and wrote the paper; All authors reviewed and approved the final manuscript.

Institutional review board statement: The study was approved from the Ethics Committee of University Witten/Herdecke and was performed in accordance with institutional guidelines. Written informed consent was waived for retrospective study participation.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: All authors declare no conflicts of interest.

Data sharing statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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S-Editor: Wang LL
L-Editor: Filipodia
P-Editor: Wang LL

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World Journal of Clinical Oncology

Submit a Manuscript: https://www.f6publishing.com

World J Clin Oncol 2022 August 24; 13(8): 712-724

DOI: 10.5306/wjco.v13.i8.712

ISSN 2218-4333 (online)

ORIGINAL ARTICLE

Prospective Study

Impact of cytochrome P450 2D6 polymorphisms on decision-making and clinical outcomes in adjuvant hormonal therapy for breast cancer

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Specialty type: Oncology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Kamalabadi-Farahani M, Iran

Received: April 7, 2021
Peer-review started: April 7, 2021
First decision: June 28, 2021
Revised: August 8, 2021
Accepted: July 18, 2022
Article in press: July 18, 2022
Published online: August 24, 2022

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Abstract

BACKGROUND

There are concerns that tamoxifen is less effective in Asian women because of the high prevalence of impaired function cytochrome P450 2D6 (*CYP2D6*) polymorphisms.

AIM

To evaluate how knowledge of *CYP2D6* genotype impacted the choice of hormonal agent and how *CYP2D6* genotype and agent were associated with clinical outcomes.

METHODS

Eighty-two women were recruited. Seventy-eight completed *CYP2D6* genotyping and were categorized into poor, intermediate (IM) and extensive or ultra metabolizer phenotypes. Women with poor metabolizer and IM phenotypes were recommended aromatase inhibitors as the preferred agent.

RESULTS

More than 70% of the women had an IM phenotype, 32% an extensive or ultra metabolizer phenotype, and 0% had a poor metabolizer phenotype. Regardless of genotype, more women opted for aromatase inhibitors. Overall, 80% of women completed 5 years of hormonal therapy. Five women developed recurrence, 3 contralateral breast cancer, 5 died, and 1 was diagnosed with a second primary cancer. Five-year recurrence-free and overall survival were slightly better in women with the extensive or ultra metabolizer phenotype compared to those with the IM phenotype, though not statistically significant [P = 0.743, hazard ratio (HR): 1.441, 95% confidence interval (CI): 0.191 to 10.17 and P = 0.798, HR: 1.327, 95%CI: 0.172 to 9.915, respectively]. Women receiving aromatase inhibitors also appeared to have a better, but also nonsignificant, 5-year recurrence-free and overall survival (P = 0.253, HR: 0.368, 95%CI: 0.031 to 0.258 and P = 0.292, HR: 0.252, 95%CI: 0.005 to 4.951, respectively).

CONCLUSION

The IM phenotype was highly prevalent but was not associated with clinical outcome.

Key Words: Functional cytochrome P450 2D6 polymorphisms; Breast cancer; Hormonal therapy

Core Tip: We studied the role of cytochrome P450 2D6 (*CYP2D6*) polymorphisms in guiding the selection of hormonal agents in women with hormone-responsive breast cancer. The CYP2D6 intermediate metabolizer phenotype was highly prevalent in our women, while the poor metabolizer phenotype was rare. We did not observe any significant association between the CYP2D6 phenotypes and recurrence-free or overall survival in our study, although it could be because most women opted for aromatase inhibitors regardless of CYP2D6 phenotype. There was a non-significant trend towards better survival associated with aromatase inhibitor use over tamoxifen.

Citation: Tan EY, Bharwani L, Chia YH, Soong RCT, Lee SSY, Chen JJC, Chan PMY. Impact of cytochrome P450 2D6 polymorphisms on decision-making and clinical outcomes in adjuvant hormonal therapy for breast cancer. *World J Clin Oncol* 2022; 13(8): 712-724

URL: https://www.wjgnet.com/2218-4333/full/v13/i8/712.htm

DOI: https://dx.doi.org/10.5306/wjco.v13.i8.712

INTRODUCTION

In many centers, aromatase inhibitors (AIs) are now the first-line adjuvant hormonal therapy agents recommended for hormone-responsive breast cancer. While there are several reports of superior efficacy with AIs[1-3], some have questioned whether this is seen only in women with impaired tamoxifen metabolism[4,5]. Tamoxifen undergoes extensive first pass oxidative metabolism by the cytochrome P450 2D6 (CYP2D6) enzyme into the metabolically active derivative endoxifen (4-hydroxy-N-desmethyl-tamoxifen)[6,7]. The effect of impaired tamoxifen metabolism has particular significance among certain patient groups including Asians, where only 50% have functional CYP2D6 alleles. The reduced function allele *CYP2D6*10* is highly prevalent among Asians and results in a 60% reduction in CYP2D6 enzyme activity[8-14]. The lower levels of the active metabolite endoxifen could imply that a large number of Asian women may have sub-therapeutic levels of tamoxifen and consequently suboptimal risk reduction. In contrast to tamoxifen, AIs act by inhibiting the aromatase enzymatic conversion of androgens to estradiol and is not affected by CYP2D6 metabolism.

At the time when this study was initiated, cost and the treatment duration were significant factors contributing to patient cost of the hormonal agent. Previously, AIs cost almost 25 times more than tamoxifen, and AIs were recommended for 5 years, whereas tamoxifen began to be recommended for 10 years. These factors are less relevant today. The cost of AIs are now relatively similar to tamoxifen with generic preparations of AIs now available, and extended AI therapy is also more often recommended. In spite of this, tamoxifen remains an important hormonal agent, particularly in premenopausal women, where the use of an AI will require concomitant use of gonadotropin-releasing hormone inhibitors. This is seldom done unless the risk of recurrence risk is high. Tamoxifen also remains a valuable alternative in women who cannot tolerate the musculoskeletal side effects of AIs or who develop osteoporosis from accelerated bone loss.

In this study, we evaluated the frequency of *CYP2D6* polymorphisms and examined how the knowledge of the CYP2D6 phenotype impacted patient's choice of hormonal agent. We also evaluated

the association with clinical outcome through endpoints such as disease recurrence, mortality, contralateral breast cancer and 5-year recurrence-free and overall survival. We also evaluated the adverse effects reported by patients and the compliance to each agent, including the frequency of a switch to an alternative agent or premature discontinuation of hormonal therapy.

MATERIALS AND METHODS

Description of study cohort and CYP2D6 phenotype classification

The study was designed as a single-arm prospective study and recruited 82 women with breast cancer. The study was granted Ethics Committee approval (2011/00017). Women included into the study were: (1) Post-menopausal; (2) Histologically confirmed with invasive breast carcinoma, Stage I to III; (3) Proven to have estrogen receptor (ER) and/or progesterone receptor (PR)-positive tumors (tumors with cells with at least 1% of cells staining positive for ER or PR were considered positive); (4) Had completed curative breast cancer surgery; (5) Had been recommended adjuvant hormonal therapy by the multidisciplinary tumor board; and (6) Were capable of providing informed consent. Exclusion criteria were: (1) Ductal carcinoma in situ; (2) Microinvasive disease; (3) Metastatic disease at presentation (including those found with metastatic disease on staging scans done after surgery); (4) Prior personal history of breast cancer or other primary cancers; and (5) Specific contraindications to tamoxifen and AIs, such as previous deep venous thrombosis, pulmonary embolism, cerebrovascular accident and severe osteoporosis.

Blood was sampled from patients who satisfied both criteria for *CYP2D6* genotyping. The Qiagen DNA extraction kit (Qiagen, Hilden, Germany) was used to extract genomic DNA from blood collected; DNA concentration, purity and integrity were verified for all samples. Three poor metabolizer (PM) alleles (*4, *5, *6) and three intermediate metabolizer (IM) alleles (*9, *10 and *41) were identified using the pyrosequencing method, as previously described[15]. These six alleles were selected based on the reported prevalence among women of Chinese and Malay ethnicity, who make up the majority of the study cohort[8-14]. Primers were designed using the pyrosequencing software (http://techsupport.pyrosequencing.com); primer sequences are listed in Table 1.

The PM phenotype was defined by being homozygous or compound heterozygous for two PM alleles. The IM phenotype was defined by being homozygous for two IM alleles or heterozygous for a PM allele and an IM allele. All other combinations are considered either extensive metabolizer (EM) or ultra metabolizer (UM) phenotypes. A PM or IM allele in combination with an EM allele results in an EM phenotype (tamoxifen metabolism being comparable). Patients were classified according to the following combinations: (1) PM: *4/*4, *4/*5, *4/*6*, *5/*5, *5/*6, *6/*6; (2) IM: *9/*9, *9/*10, *9/*41, *10/*10, *10/*41, *41/*41, *9/*4, *9/*5, *9/*6, *10/*4, *10/*5, *10/*6, *41/*4, *41/*5, *41/*6, heterozygotes for *4, *5, *6, *9, *10, *41; (3) EM/UM: all other combinations.

Hormonal therapy recommendations and outcomes based on CYP2D6 genotyping

Results of the *CYP2D6* genotyping were made known to the patient during the discussion for hormonal therapy. Patients identified to have PM or IM phenotypes were recommended anastrozole at a dose of 1 mg daily or letrozole at a dose of 2.5 mg daily, but they were still permitted to opt for tamoxifen. Those with an EM/UM phenotype were given a choice between an AI or tamoxifen (20 mg daily). All agents were recommended for a duration of 5 years, which was the standard practice at the time of the study. The benefit and potential treatment-related side effects of either agent was discussed with the patient, and the women themselves made the final decision regarding the choice of hormonal agent. The women were assessed every 6 mo with clinical history and physical examination during regular surveillance visits, and any side effects or disease progression were documented. Women receiving AIs also received calcium and vitamin D supplements, and bone mineral density was monitored every year. Women receiving tamoxifen were referred to the gynecology clinic for surveillance that included a yearly ultrasound of the pelvis to evaluate endometrial thickness specially; this was in addition to the routine 3-yearly PAP smear screenings. While this study was originally designed to follow up for the 5-year duration of hormonal treatment, we continued to collect data from the women who remained on extended therapy and on follow-up with the breast clinic in view of the increasing use of hormonal agents beyond 5 years as the study progressed. All 70 women included in the final analyses remained on follow-up until study completion. Median follow-up was 86 mo (30.57 to 99.50 mo), and median overall survival rate was 75.95 mo (30.57 to 94.10 mo).

Statistical analyses

The associations between CYP2D6 phenotype and the specific hormonal agent received and with standard clinicopathological parameters and outcomes were evaluated with univariate analyses (χ^2 test, Fisher's test, one-way analysis of variance, χ^2 test for trend for ordinal data) and were performed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA, United States). Recurrence-free survival was defined as the time interval from surgery to the development of either locoregional or distant

Table 1 Primers for cytochrome P450 2D6 genotyping

	Position/change	Forward primer (5'→3')	Reverse primer (5'→3')
*4	1846G>A	AGA GGC GCT TCT CCG TGT CC	AAA TCC TGC TCT TCC GAG GC
*5	Gene deletion	CTC CAG CCT CCA CCA GTC CAG	CAG GCA TGA GCT AAG GCA CCC AGA C
*6	1707delT	CGC AAC TTG GGC CTG GGC AAG AAG TCG CTG GAC TAG	CTC GGG AGC TCG CCC TGC AGA GAC TC
*9	2613AGA>del	GGT CAG TGG TAA GGA CAG GCA GGC CC	CAC CCT TGC CCC CCA CCG TGG CAG CCA CTC TAA GCT
*10	100C>T	GAT GCA CCG GCG CCA ACG CTG GGC TGC ACG GTA C	CAA ACC TGC TTC CCC TTC TCA GCC
*41	2988G>A	CGT GAG CCC ATC TGG GAA A	CTG ACA CTC CTT CTT GCC TCC TA

recurrence. Overall survival was defined as the time interval from surgery to death, whether from breast cancer specific mortality or from any other causes. Contralateral cancer was defined as the occurrence of a metachronous cancer in the contralateral breast more than 6 mo following the diagnosis of the first cancer. Kaplan Meier survival curves were performed to compare survival rates, and the log rank test was used to compare between the two arms. A two-tailed test was used for all analyses, and a value of $P < 0.05$ was considered statistically significant.

RESULTS

Study cohort demographics

Over a 3-year period from 2011 to 2013, a total of 82 women were recruited into the study. Eleven women later opted to withdraw for personal reasons, and one woman was withdrawn after she developed metastatic disease while on adjuvant chemotherapy treatment (prior to the start of hormonal treatment). *CYP2D6* genotyping was completed in 78 women, and study endpoints were evaluated in the 70 women who remained in the study and completed at least 5 years of follow-up (apart from 3 women who died). Median patient age was 61 years (ranging from 47 years to 86 years), and the majority (70 of 82, 85.4%) were of Chinese ethnicity. More than half (62 of 82, 75.6%) of the women had at least one pre-existing co-morbidity; hypertension was the most common. All the women had undergone curative surgery, with 64.6% having had a mastectomy. Disease was staged as Stage I in 34 of 82 women (41.5%), Stage II in 27 (32.9%) and Stage III in 21 (25.6%); 40.2% of women had node-positive disease. Invasive ductal carcinoma not otherwise specified was the most common histological type (78%). Median invasive tumor size was 2 cm (ranging from 0.1 to 8.0 cm), and median tumor grade was 2.

All but 2 women had ER-positive tumors; they had ER-negative/PR-positive tumors. Median ER staining intensity was strong, and the median proportion of tumor cells staining positive for ER was 90.7%. Details of tumor ER expression is as follows: less than 10% of tumor cells stained positive in 4 of 80 women (4.9%), 11% to 49% of cells stained positive in 8 women (8.9%), 50% to 89% of cells stained positive in 17 women (24.3%) and more than 90% of cells stained positive in 41 women (58.7%). Tumors were positive for PR in 61 women (87.1%) and human epidermal growth factor receptor-2-positive in 31 (44.3%) women.

The majority of women (73.1%) were classified as having an IM phenotype, 25 women (32.1%) as having an EM/UM phenotype, and none were classified as having a PM phenotype (Table 2). The *10 allele was highly prevalent and was found in 52 (66.7%) women. Another 2 (2.5%) women were found with the *41 allele. The *4 and *5 PM alleles were found in 5 (6.4%) women, and in all instances occurred together with a *10 allele. Of the 8 women who withdrew from the study and who were not included in endpoint analyses, 5 were of the IM phenotype and 3 were of the EM/UM phenotype. Women with IM and EM/UM phenotypes shared similar characteristics (Table 3). Specifically, the IM phenotype did not correlate with tumor receptor status nor with the intensity of ER staining or proportion of tumor cells staining positive for ER.

Details of hormonal agent choice and treatment

Following a discussion with their attending clinician, more women opted for an AI over tamoxifen regardless of CYP2D6 phenotype. More than 80% of the women (43 of 52, 82.7%) with an IM phenotype opted for an AI, and 72.2% of women with an EM/UM phenotype (13 of 18) also opted for an AI. Only 14 women (9 with an IM phenotype and 5 with an EM/UM phenotype) opted for tamoxifen. There were no significant differences between the group who opted for an AI compared to those who chose

Table 2 Details of cytochrome P450 2D6 genotyping for the 78 women classified into two phenotype groups: Intermediate metabolizers and extensive metabolizers/ultra metabolizers

Phenotype	CYP2D6 genotype	Number of women, *n* = 78
IM	*10/*10	25
	*10/41	2
	*10/*4	2
	*10/*5	3
	*10/EM/UM	20
	*5/EM/UM	5
EM/UM	EM/UM	21

IM: Intermediate metabolizers; EM/UM: Extensive metabolizers/ultra metabolizers; CYP2D6: Cytochrome P450 2D6.

tamoxifen (Table 4). The difference in median ages between the two groups (P = 0.008) was not likely clinically significant. Similar numbers of women on AI and tamoxifen choose mastectomy, but more women opting for AI had received other modes of systemic treatment (chemotherapy with or without trastuzumab) (P = 0.045).

Overall, 56 of the 70 women (80%) completed 5 years of hormonal therapy; comprising 46 of the 56 women (82.1%) who opted for an AI and 10 of the 14 women (71.4%) who opted for tamoxifen. Nineteen women who started on an AI and three of those who started on tamoxifen continued with extended therapy after 5 years of treatment. Thirty of the 56 women (53.5%) completed 5 years of the initial AI agent they were started on. Side effects were reported in 19 women: severe myalgia and arthralgia in 8 women, skin rashes in 4 women and osteoporosis in 7 women. Two women with intolerable musculoskeletal side effects opted to discontinue hormonal therapy. Of the other 12 women with musculoskeletal side effects or rashes, 7 switched to another AI, and 3 switched to tamoxifen. Four of the seven women who developed osteoporosis switched to tamoxifen, while the remaining three remained on AIs but started on bisphosphonates. All the women who switched to another AI agent eventually completed 5 years of treatment, although 2 switched to a third AI agent and 3 switched to tamoxifen before completion. Of the 7 women who switched to tamoxifen, 6 completed 5 years of treatment with tamoxifen, while the remaining patient switched back to another 2 different AI agents before completing the 5 years of treatment. Of the 14 women who started on tamoxifen, 2 women (14.3%) switched to an AI after developing skin rashes; though one of them later discontinued the AI after developing musculoskeletal side effects. The hormonal agent was discontinued in 8 patients upon the development of new events. Another 3 women chose to discontinue hormonal therapy but had not reported any side effects.

Clinical outcomes

Over the follow-up period of 96 mo, 3 women developed contralateral breast cancer, 5 women developed recurrences, 5 women died, and 1 woman was diagnosed with a nasopharyngeal carcinoma. Contralateral breast cancer was diagnosed after a median interval of 48.8 mo (ranging from 47.83 to 59.27 mo). All 3 occurred in women of the IM phenotype, of which 2 women had received AI and 1 received tamoxifen. Recurrence was systemic in all 5 women and had occurred after a median interval of 24.93 mo (ranging from 23.07 to 48.10 mo). Four of these women later died, although one death was attributed to a non-breast cancer-related cause. The last mortality occurred in a woman who had remained disease-free up to the time of death from a non-breast cancer-related cause. Disease recurrence did not show a clear association with the hormonal agent received (P = 0.260) (Table 4). Recurrence developed in 3 women who had received an AI (2 of the IM phenotype and 1 of the EM/UM phenotype) and in 2 women who had received tamoxifen (both of the IM phenotype). One of the women developed both locoregional and systemic recurrence.

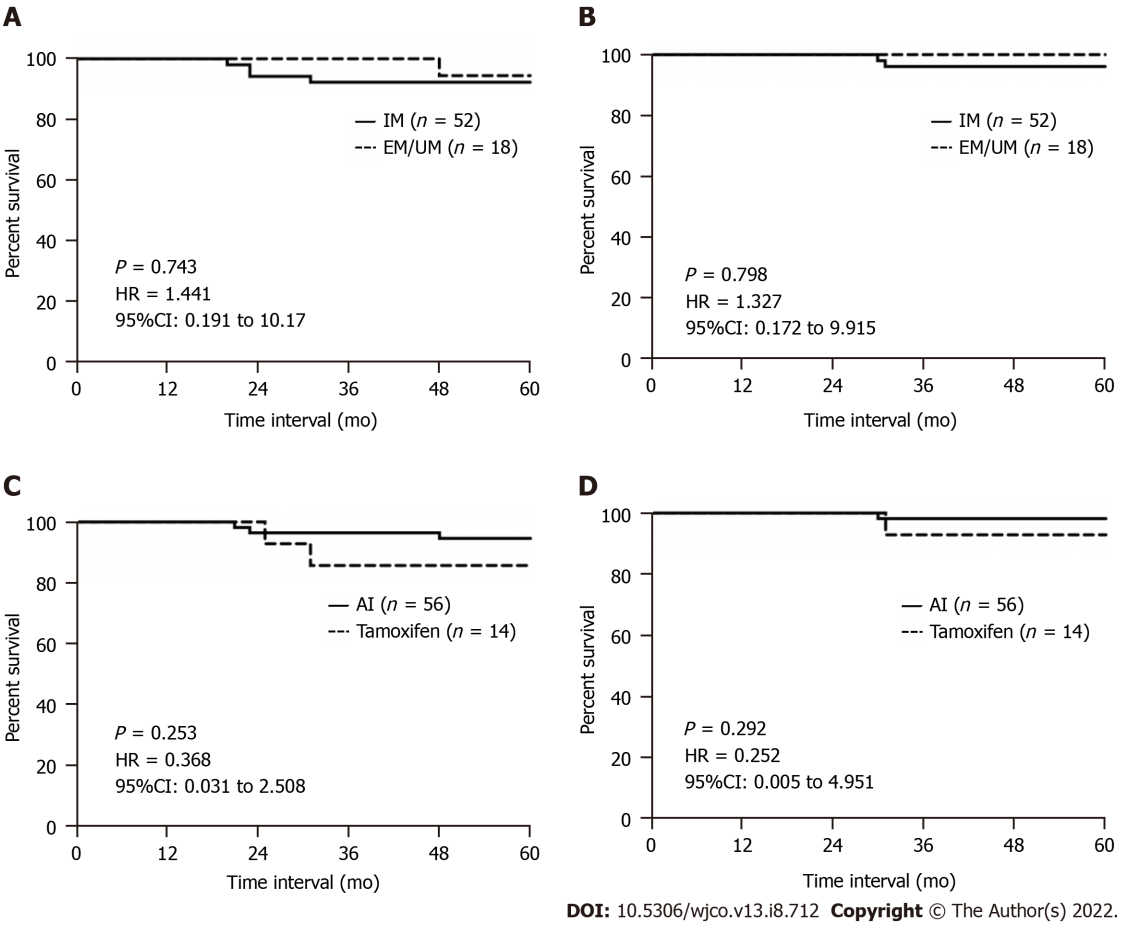
Five-year recurrence-free and overall survival appeared slightly better in women with an EM/UM phenotype compared to those with an IM phenotype but was not statistically significant [P = 0.743, hazard ratio (HR): 1.441, 95% confidence interval (CI): 0.191 to 10.17 and P = 0.798, HR: 1.327, 95%CI: 0.172 to 9.915, respectively) (Figure 1A and B). When stratified by the hormonal agent received, women who had received an AI appeared to have better 5-year recurrence-free and overall survival compared to those who had received tamoxifen, but again these were not statistically significant (P = 0.253, HR: 0.368, 95%CI: 0.031 to 0.258 and P = 0.292, HR: 0.252, 95%CI: 0.005 to 4.951, respectively) (Figure 1C and D).

Table 3 Univariate correlation analyses of clinicopathological parameters with cytochrome P450 2D6 phenotype classification

	IM phenotype, *n* = 52	EM/UM phenotype, *n* = 18	*P* value
Median age in yr	62.5 (51-86)	61 (50-84)	0.574
Disease stage			0.595
I	24	6	
II	17	8	
III	11	4	
Ethnicity			0.148
Chinese	47	13	
Malay	3	3	
Indian	1	2	
Others	1	0	
Comorbidities			0.728
Yes	43	14	
No	9	4	
Tumor histology			0.518
IDC	40	14	
ILC	7	1	
Others	5	3	
Tumor grade			0.247
1	16	3	
2	25	10	
3	11	5	
Median tumor size in mm	16.5 (1.2 to 70.0)	20.0 (3.0 to 45.0)	0.334
Lymphovascular invasion			0.527
Present	16	7	
Absent	36	11	
ER intensity			0.528
Low	2	0	
Moderate	11	2	
High	38	15	
Negative	1	1	
Proportion of tumor cells staining ER-positive			0.267
1% to 10%	4	0	
11% to 49%	6	1	
50% to 89%	14	3	
More than 90%	27	14	
PR intensity			0.631
Low	5	1	
Moderate	9	5	
High	31	11	
Negative	7	1	
Proportion of tumor cells staining PR-positive			0.785

1% to 10%	11	3	
11% to 49%	8	2	
50% to 89%	18	7	
More than 90%	11	6	
HER2 status			0.495
Positive	11	2	
Negative	41	16	
Clinical subtypes			0.692
ER+/HER2-	41	14	
ER+/HER2+	10	2	
ER-/HER2+	1	0	

ER: Estrogen receptor; PR: Progesterone receptor; HER2: Human epidermal growth factor receptor-2; IDC: Invasive ductal carcinoma; ILC: Invasive lobular carcinoma; IM: Intermediate metabolizers; EM/UM: Extensive metabolizers/ultra metabolizers.



DOI: 10.5306/wjco.v13.i8.712

Figure 1 Survival outcomes stratified by cytochrome P450 2D6 phenotype and by hormonal agent used. A: Kaplan-Meier curves showing 5-year recurrence-free survival stratified by cytochrome P450 2D6 phenotype (n = 70); B: Kaplan-Meier curves showing 5-year overall survival stratified by cytochrome P450 2D6 phenotype (n = 70); C: Kaplan-Meier curves showing 5-year recurrence-free survival stratified by hormonal agent received (n = 70); D: Kaplan-Meier curves showing 5-year overall survival stratified by hormonal agent received (n = 70). IM: Intermediate metabolizers; EM/UM: Extensive metabolizers/ultra metabolizers; AI: Aromatase inhibitors; HR: Hazard ratio; CI: Confidence interval.

DISCUSSION

AIs are now widely used as first-line hormonal agent in many clinical units. With the introduction of generic letrozole, AIs have become more affordable for our local women. The common practice here is to start women on AIs unless they have contraindications or develop intolerable side effects. Tamoxifen

Table 4 Univariate correlation analyses of clinicopathological parameters and clinical outcome with type of hormonal agent received

	Aromatase inhibitors, *n* = 56	Tamoxifen, *n* = 14	*P* value
CYP2D6 phenotype			0.339
IM	43	9	
EM/UM	13	5	
Median age in yr	62 (50 to 80)	63 (52 to 86)	0.008
Disease stage			0.795
I	23	7	
II	21	4	
III	12	3	
Ethnicity			0.126
Chinese	50	10	
Malay	3	2	
Indian	3	1	
Others	0	1	
Comorbidities			1.000
Yes	45	12	
No	11	2	
Tumor histology			0.918
IDC	45	11	
ILC	6	2	
Others	5	1	
Tumor grade			0.686
1	15	4	
2	27	8	
3	14	2	
Median tumor size in mm	16.5 (1.2 to 70.0)	19.0 (1.6 to 53.0)	0.747
Lymphovascular invasion			0.526
Yes	17	6	
No	39	8	
ER intensity			0.509
Low intensity	1	1	
Moderate intensity	10	4	
High intensity	44	9	
Negative	1	0	
Proportion of tumor cells staining ER-positive			0.386
1%-10%	3	1	
11% to 49%	4	3	
50% to 89%	15	2	
More than 90%	33	8	
PR status			1.000
Positive	49	13	
Negative	7	1	

PR intensity			0.161
Low intensity	3	3	
Moderate intensity	10	4	
High intensity	36	6	
Negative	7	1	
Proportion of tumor cells staining PR-positive			0.318
1%-10%	11	3	
11% to 49%	6	4	
50% to 89%	21	4	
More than 90%	15	2	
HER2 status			0.700
Positive	10	3	
Negative	46	10	
Tumor subtypes			0.754
ER+/HER2-	46	10	
ER+/HER2+	9	3	
ER-/HER2+	1	0	
Type of surgery			0.756
Mastectomy	35	10	
Wide local excision	21	4	
Treatments received			0.045
Systemic therapy[1] and hormonal therapy	12	2	
Systemic therapy[1], radiation and hormonal therapy	18	0	
Radiation and hormonal therapy	12	5	
Hormonal therapy alone	14	7	
Disease recurrence[2]			0.260
Yes	3	2	
No	53	12	
Mortality[2]			0.344
Yes	1	1	
No	55	13	
Contralateral breast cancer[2]			0.551
Yes	2	1	
No	54	13	

[1]Systemic therapy refers to chemotherapy with or without trastuzumab.
[2]Events occurring within 5 years.
ER: Estrogen receptor; PR: Progesterone receptor; HER2: Human epidermal growth factor receptor-2; IDC: Invasive ductal carcinoma; ILC: Invasive lobular carcinoma; IM: Intermediate metabolizers; EM/UM: Extensive metabolizers/ultra metabolizers; CYP2D6: Cytochrome P450 2D6.

is now first-line only in premenopausal women or in those with contraindications to AIs. Otherwise, it is often the second-line agent that women are switched to should they develop intolerable side effects from tamoxifen. The move towards adopting AIs as first-line came following reports of superior efficacy and because AIs were previously recommended for 5 years, whereas tamoxifen was recommended for 10 years. However, it has also been suggested that AIs are superior to tamoxifen primarily in women with nonfunctional or reduced function *CYP2D6* polymorphisms[4,5], specifically in those with a PM or IM phenotype where impaired CYP2D6 enzyme metabolism results in lower serum concentrations of

active metabolite endoxifen responsible for tamoxifen efficacy[16,17]. Observations that serum endoxifen levels correlated with the frequency and severity of adverse effects raised the possibility that clinical outcomes could likewise be adversely affected by impaired CYP2D6 metabolism[18,19].

More than 70% of Caucasians have functional *CYP2D6* alleles, yet only 50% of Asians have functional *CYP2D6* alleles[8-10]. It has been variously reported that 40% to 70% of Asians carry reduced function alleles, particularly the *CYP2D6*10* allele[11-14]. The high prevalence of *CYP2D6*10* was confirmed in our study, where it was present in two-thirds of the women included. The *41 allele was present in 2 women. The *CYP2D6*10* allele results in an approximately 60% reduction in CYP2D6 enzyme activity, and Asians have been reported to metabolize tamoxifen and other CYP2D6-mediated drugs more slowly than Caucasians[20-23]. Nonfunctional alleles were uncommon, and all 5 women (6.4%) in our study with *4 and *5 PM alleles were heterozygotes. This is relatively similar to the prevalence reported in other studies[9-11,13,14,22,23]. We did not find any women with the *CYP2D6*9* allele, which we had included as it was observed in 3% of Malays in Malaysia[9].

Five women (7.1%) in our study developed recurrence during the study, including 4 women who were of the IM phenotype; 2 of whom received an AI. We did observe a trend towards better survival in women with the EM/UM phenotype. This was in agreement with the findings of a prospective study window-of-opportunity study conducted at our unit where women received up to 2 wk of tamoxifen prior to surgery. Women with at least one wild-type *CYP2D6* allele demonstrated a significantly greater Ki-67 response, suggesting that tamoxifen produced a greater inhibitory effect in those with functioning *CYP2D6* polymorphisms[24]. The lack of statistical significance was likely because of the small sample size and perhaps because there were no women with the PM phenotype in our study. Women with a PM phenotype were reportedly at a 7% higher risk of recurrence, which appeared to be incremental over time[5]. Like others, we did not observe *CYP2D6* polymorphisms to correlate with tumor size, grade or nodal status, which meant that survival differences were not likely a result of unfavorable tumor factors[25].

Mathematical modelling showed that survival outcomes in women without nonfunctional alleles were not different whether they received AIs or tamoxifen[5], and reduced function *CYP2D6* polymorphisms were only associated with clinical outcomes in those treated with tamoxifen[25,26]. We observed that women who received AIs had a slightly better but nonsignificant survival and that these women were more likely to have received systemic treatments (chemotherapy with or without trastuzumab), perhaps indicating a clinician bias towards AIs in those deemed to have more 'high-risk' disease. However, our study numbers are small and too few women received tamoxifen for a meaningful analysis. A larger cohort would be needed to stratify the effect of the hormonal agent used by disease stage and other systemic treatments. Overall, we observed little difference in contralateral breast cancer rates and disease recurrence between women with IM and EM/UM phenotypes, which was perhaps due to many women in our study, including 72% of those with an EM/UM phenotype, opting for an AI over tamoxifen.

Despite the differences in cost at the time of the study, many women opted for the more costly AI. This was so even in those with an EM/UM phenotype, in whom there were suggestions that tamoxifen efficacy was comparable to AI. However, only about half of the women in our study completed 5 years of the AI agent they were initially started on. In 60% of cases, the discontinuation was initiated by the women themselves because of intolerable myalgia, arthralgia and skin rashes [10 (53%) switched to another AI, and 5 had further problems with the second AI and eventually switched to tamoxifen or a third AI]. Four women who started on an AI were switched to tamoxifen by their clinician because of osteoporosis from accelerated bone loss. On the other hand, the majority of women who started on tamoxifen appeared to tolerate it well, and only 2 were switched to an AI after developing skin rashes. Overall, more women who started on tamoxifen completed 5 years of treatment compared to those who started on an AI. Excluding the 8 women who progressed on hormonal therapy, 90% of the women completed 5 years of hormonal therapy.

Like others, we did not find *CYP2D6* polymorphisms to correlate with any clinicopathological factors, implying that genotyping would be the only means of ascertaining the phenotype. The prevalence of the IM phenotype in our local women may mean that *CYP2D6* genotyping at the offset may be of little benefit since AIs are now the initial hormonal agent of choice. On the other hand, given that a significant number of women do develop AI-related side effects and in those whom a switch is being considered, *CYP2D6* genotyping could help clinicians decide whether to switch to another AI or to tamoxifen. Those with an EM/UM phenotype can be switched to tamoxifen since outcomes are probably comparable with those on AIs[5]. Furthermore, tamoxifen-related side effects appear to be less common. An unpublished review of women on follow-up at our unit did not find a higher incidence of endometrial cancer among those treated with tamoxifen. Those with an IM phenotype should consider switching to another AI agent, based on reports of impaired function variants being associated with higher risks of recurrence. This would have particular significance in the setting of ER-positive disease where late recurrences are more common and since the majority of women survive for many more years after breast cancer treatment.

CONCLUSION

The prevalence of the IM phenotype was high in our study, with more than two-thirds of the women having the *CYP2D6*10* allele. We did not observe the IM phenotype to be associated with any clinicopathological parameter and did not observe any correlation with clinical outcome. The hormonal agent used was not associated with a difference in outcome. Compliance was good, and most women completed 5 years of hormonal therapy, although more women who started on an AI required a switch to another hormonal agent because of side effects.

ARTICLE HIGHLIGHTS

Research background

There are concerns that tamoxifen is less effective in Asian women because of the high prevalence of impaired function cytochrome P450 2D6 (*CYP2D6*) polymorphisms.

Research motivation

Tamoxifen is still the first-line agent for premenopausal women and for those with intolerable AI-related side effects. It is therefore necessary to verify the effectiveness of tamoxifen in view of the high prevalence of reduced function *CYP2D6* polymorphisms in Asians.

Research objectives

We evaluated the frequency of *CYP2D6* polymorphisms and its association with clinical outcome. We also evaluated treatment-related side effects in order to better determine the risk:benefit ratio.

Research methods

We designed a single-arm prospective study to evaluate how knowledge of *CYP2D6* genotype impacted the choice of hormonal agent and how *CYP2D6* genotype and agent were associated with clinical outcomes.

Research results

More than 70% of the women in our study had an intermediate metabolizer phenotype. Regardless of genotype, more women opted for aromatase inhibitors. Women with the extensive or ultra metabolizer phenotype had slightly better but nonsignificant 5-year recurrence-free and overall survival compared to women with the intermediate metabolizer phenotype. Women on AIs appeared to have better but also nonsignificant 5-year recurrence-free and overall survival.

Research conclusions

The intermediate metabolizer phenotype was highly prevalent in our local women but was not associated with clinical outcome.

Research perspectives

Data on the effect of *CYP2D6* polymorphisms on tamoxifen efficacy remains conflicting. More studies in Asian women would help to clarify this association.

ACKNOWLEDGEMENTS

The authors are grateful to Ms Sim E-Jan for contributions to the generation and presentation of Figure 1.

FOOTNOTES

Author contributions: Tan EY designed and performed the research study, analyzed the data and wrote the manuscript; Bharwani L and Chia YH performed study procedures in the protocol; Soong RCT was responsible for *CYP2D6* genotype and verification of the phenotype; Lee SSY, Chen JJC and Chan PMY contributed to data collection, data analyses and review of the manuscript; All authors have read and approved the final manuscript.

Supported by the NHG-KTPH Small Innovative Grants (SIG), No. SIG/11009 and No. SIG/15025.

Institutional review board statement: This study has obtained Ethics Committee approval (2011/00017).

Clinical trial registration statement: This study is registered at the Health Sciences Authority (Application No. 1140684B).

Informed consent statement: Documented consent was obtained from all patients recruited into this study for the use of blood samples and genomic and clinical data.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at Ern_Yu_Tan@ttsh.com.sg. Participants gave informed consent for data sharing.

Country/Territory of origin: Singapore

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S-Editor: Gong ZM
L-Editor: Filipodia
P-Editor: Gong ZM

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World Journal of Clinical Oncology

Submit a Manuscript: https://www.f6publishing.com

World J Clin Oncol 2022 August 24; 13(8): 725-728

DOI: 10.5306/wjco.v13.i8.725

ISSN 2218-4333 (online)

LETTER TO THE EDITOR

COVID-19 and oral cancer: Critical viewpoint

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Specialty type: Cell biology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Balaban DV, Romania

Received: April 9, 2021
Peer-review started: April 9, 2021
First decision: June 28, 2021
Revised: July 2, 2021
Accepted: July 25, 2022
Article in press: July 25, 2022
Published online: August 24, 2022

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Abstract

The outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has marked the beginning of a new pandemic named coronavirus disease 2019 (COVID-19). The World Health Organization has announced it as a health emergency that is of international concern. The disease has been reported to cause respiratory illness, pneumonia and even hinder the immunity of an individual. Individuals with disturbed immune responses have been found to be quite susceptible to this viral infection. Oral cancer patients are also at high risk in this pandemic situation and might encounter severe detrimental outcomes. Angiotensin receptors, documented in studies as the path of entry of this virus, are highly expressed in the epithelial cells of oral mucosa, making the group of individuals with oral cancers even more vulnerable. Extracellular matrix metalloproteinase inducer is another potential target for SARS-CoV-2. An exhaustion of angiotensin converting enzyme 2 cell receptors leads to protumoral effects, whereas a downregulation of extracellular matrix metalloproteinase inducer leads to antitumoral effects. Thus, it causes a variation of the biological behavior of the tumor. This article focusses on the molecular mechanisms, effects and pathophysiology of COVID-19 in oral squamous cell carcinoma patients. The different molecular changes in oral squamous cell carcinoma in the background of COVID-19 will modify various environmental factors for this pathology and have an effect on the carcinogenesis process. Understanding the behavior of the tumor will help plan advanced treatment strategies for oral squamous cell carcinoma patients in the background of COVID-19.

Key Words: COVID-19; SARS-CoV-2; Oral cancer; Head and neck carcinomas; Oral squamous cell carcinoma

Core Tip: The outbreak of coronavirus disease 2019 (COVID-19) has evoked concern worldwide. The rapid spread of the disease during the first and the second waves caused severe respiratory illness. Individuals are facing a suppressed immune response. An impaired immune response has made patients with head and neck cancer highly susceptible to the viral infection. The two potential receptors of severe acute respiratory syndrome coronavirus 2, angiotensin receptors and extracellular matrix metalloproteinase inducer, have contrasting effects on cancer progression. Thus, the molecular mechanisms and the biological behavior of oral squamous cell carcinoma show varying effects in the background of COVID-19.

Citation: Gopalakrishnan D, Sarode SC, Sarode GS, Sengupta N. COVID-19 and oral cancer: Critical viewpoint. *World J Clin Oncol* 2022; 13(8): 725-728
URL: https://www.wjgnet.com/2218-4333/full/v13/i8/725.htm
DOI: https://dx.doi.org/10.5306/wjco.v13.i8.725

TO THE EDITOR

The outbreak of coronavirus disease 2019 (COVID-19) has posed a major health impact, affecting populations all over the world with significant morbidity and mortality. With the introduction of the second wave in many countries, the doubling time of infectivity has reduced drastically. This also means that we should doubly prepare for all the consequences that we faced in the first wave. Individuals with disturbed immune responses have been found to be quite susceptible to this viral infection. Cancer patients have been considered to be at high risk in this pandemic situation because of immunosuppression[1]. Not only the underlying malignant condition but also co-morbidities, advanced age and poor host response have been held responsible for the vulnerability of cancer patients during the COVID-19 pandemic[2,3].

Studies have identified the angiotensin converting enzyme 2 (ACE2) cell receptors as the path of entry of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) into a host cell[4]. ACE2 receptors are reportedly found to be highly expressed on the epithelial cells of oral mucosa making the group of individuals with oral cancers even more vulnerable. ACE2, a key enzyme of renin-angiotensin system, breaks down angiotensin II (Ang II) into Ang 1-7[5]. Ang II is a protumoral agent that plays a major role in carcinogenesis[6]. It helps in tumor cell proliferation and angiogenesis. It also facilitates the metastasis of cancer cells. Thus, Ang II aids in progression of the disease, while ACE2 and Ang 1-7 inhibit the progression. ACE2 maintains a balance of renin-angiotensin system[5]. However, these propositions might alter due to changes in the viral component, specially mutation in the spike protein.

The SARS-CoV-2 attaches to ACE2 cell receptors through the S-spikes on the virus surface. The SPIKE (S protein) expressed by the virus attaches to the extracellular part of ACE2 receptors, and the S protein breaks down into subunits S1 and S2[7]. The virus fuses with the cell membrane and gains entry into the cell *via* endocytosis. An exhaustion of ACE2 receptors takes place due to the viral infection. ACE2 receptors being highly expressed in tongue, gingiva and buccal epithelial cells, oral squamous cell carcinoma (OSCC) patients are at high risk during this pandemic[7]. The viral infection will cause a reduction in ACE2 concentration leading to an increase in Ang II concentration[5]. This could have a protumoral effect facilitating the progression of OSCC.

Besides ACE2 receptors, extracellular matrix metalloproteinase inducer (EMMPRIN), also known as BASIGIN/CD147 has been identified as another potential target for SARS-CoV-2[8]. EMMPRIN is a cell surface glycoprotein belonging to the immunoglobulin family. It helps in activation of molecules of several matrix metalloproteinases. Thus, it helps in proliferation of tumor cells and their invasion and migration[9]. EMMPRIN also promotes angiogenesis by stimulating vascular endothelial growth factors in the tumor microenvironment[10]. It is speculated that EMMPRIN expression is increased in oral carcinogenesis. The upregulation of EMMPRIN expression in OSCC patients might make them more susceptible to COVID-19 infection[8]. The virus attaches itself to EMMPRIN receptors through S receptors; thus, COVID-19 in OSCC patients will lead to downregulation of EMMPRIN receptors. This will inhibit the progression of the tumor due to scarcity of EMMPRIN receptors.

The COVID-19 infection in OSCC patients will reduce the availability of ACE2 receptors. This will lead to upregulation of Ang II concentration, thus promoting carcinogenesis. In such situations of nonavailability of ACE2 receptors, SARS-CoV-2 attaches to its next potential target, EMMPRIN receptors, to gain entry into the host cells[8]. This in turn causes downregulation of EMMPRIN receptors leading to antitumoral effects. The two potential receptors of SARS-CoV-2 have contrasting effects on OSCC progression.

COVID-19 infections in OSCC patients modulate the events of carcinogenesis and control the biological behavior of the tumor. Future molecular studies are required to have a better insight into the role of the two receptors in the pathophysiology of OSCC. Moreover, angiotensin converting enzyme

inhibitors and angiotensin receptor blockers, which are administered in cancer patients, have been thought to have varying effects on tumor progression. The use of these drugs in OSCC patients during this pandemic still remains doubtful and requires clinical studies.

The expression of ACE2 in various pathologies like oral cancer, oral submucus fibrosis and periodontitis modulate their disease process. The biological behavior of not only OSCC, but also other oral potentially malignant disorders, in the background of COVID-19 requires in-depth studies and research. This can only be achieved by representative clinical material, *i.e.* COVID-19 positive OSCC patients, appropriate disease model and their long-term follow-up. While keeping these interactions in mind, one should not forget the delay in cancer treatment worldwide. The COVID-19 pandemic has caused deviation of attention from many medical emergencies; cancer and OSCC is not an exception to it. Thus, it is mandatory to formulate guidelines for safe and effective delivery of therapeutics to cancer patient in this difficult time.

Impact of the pandemic on cancer management

Due to mandatory lockdowns during the pandemic, many healthcare specialty services were affected including cancer management. Many countries reported more than 50% reductions in the registration of new cancer patients[11]. These repercussions of the pandemic are mainly related to travel restrictions, conversion of hospitals to COVID-19 centers, fear in the mind of patients, human resource shortages, *etc.* To mitigate the reduction in the number of cases many cancer hospitals have started telecommunication and teleconsultations, but it is premature to comment on its effectiveness especially for head and neck cancer.

Due to compromised primary medical and dental services across the world, the early detection of oral cancer is at stake. Already, head and neck cancers are detected at advanced stages; further delay in the detection would lead to extremely poor prognoses. According to one study in the United States, there was a 25% reduction in newly diagnosed oral cancer cases[12]. Currently, COVID-19 is at declining stages in many countries, and this opportunity should be exploited to perform maximum screening for early detection.

FOOTNOTES

Author contributions: All the authors have contributed equally to the manuscript.

Conflict-of-interest statement: All the authors associated with the present manuscript declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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S-Editor: Gong ZM
L-Editor: Filipodia
P-Editor: Gong ZM

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