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Sex-Biased Molecular Signature for Overall Survival of Liver Cancer Patients

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Abstract

Sex/gender disparity has been shown in the incidence and prognosis of many types of diseases, probably due to differences in genes, physiological conditions such as hormones, and lifestyle between the sexes. The mortality and survival rates of many cancers, especially liver cancer, differ between men and women. Due to the pronounced sex/gender disparity, considering sex/gender may be necessary for the diagnosis and treatment of liver cancer. By analyzing research articles through a PubMed literature search, the present review identified 12 genes which showed practical relevance to cancer and sex disparities. Among the 12 sex-specific genes, 7 genes (*BAP1*, *CTNNB1*, *FOXA1*, *GSTO1*, *GSTP1*, *IL6*, and *SRPK1*) showed sex-biased function in liver cancer. Here we summarized previous findings of cancer molecular signature including our own analysis, and showed that sex-biased molecular signature $CTNNB1^{High}$, $IL6^{High}$, $RHOA^{High}$ and $GLIPR1^{Low}$ may serve as a female-specific index for prediction and evaluation of OS in liver cancer patients. This review suggests a potential implication of sex-biased molecular signature in liver cancer, providing a useful information on diagnosis and prediction of disease progression based on gender.

Key Words: Liver cancer, Sex/gender, Overall survival, Gene expression, Molecular signature

INTRODUCTION

Accumulating data demonstrate that the incidence and prognosis of many types of diseases differ depending on patients' sex/gender. The sex/gender disparity may be due to genetic differences on the X chromosome, differences in physiological conditions such as hormone levels, and other factors (Spatz *et al*., 2004; Bottarelli *et al*., 2007; Scosyrev *et al*., 2009; Klinge, 2012; Gabriele *et al*., 2016). It should be noted that 'sex' and 'gender' are not mutually exclusive terms (Clayton and Tannenbaum, 2016; Heidari *et al*., 2016). In general, 'sex' is used for biological expression, such as gene expression or hormone-related symptoms, while 'gender' is used when lifestyle, behavior, and environment are reflected (Clayton and Tannenbaum, 2016; Heidari *et al*., 2016; Pelletier *et al*., 2016). Sex/gender-biased diseases include autoimmune diseases, neurodegenerative diseases, and cardiovascular diseases (Skavdahl *et al*., 2005; Cantuti-Castelvetri *et al*., 2007; Gleicher and Barad, 2007; Haley *et al*., 2010). Diseases showing sex/gender disparities also include several types of cancers, including liver cancer, melanoma, and thyroid cancer (Bray *et al*., 2018). Liver cancer and melanoma have lower incidence rates and better prognosis in females than in males (Bray *et al*., 2018; Smalley, 2018). In contrast, the incidence rate of thyroid cancer is three times higher in females than in males, even though there is not much difference in mortality

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rate (Bray *et al*., 2018).

Liver cancer, the fourth leading cause of cancer death worldwide (Bray *et al*., 2018), shows a prominent sex/gender disparity. For liver cancer, the incidence is 3-5 times higher in males, while the mortality rates is twice as high in males than in females (Bray *et al*., 2018; Jung *et al*., 2019). The reasons for this sex/gender disparity are complex. Sex hormones, metabolic factors, and behavioral factors have been suggested as risk factors for liver cancer (Lin *et al*., 2013; Welzel *et al*., 2013; Kohi, 2016). Due to the pronounced sex/gender disparity, considering sex/gender may be necessary for the diagnosis and treatment of liver cancer (Guy and Peters, 2013).

Cancer is a complex disease and cancer genes do not act separately and deregulation of various genes from different pathways can lead to cancer initiation or progression (Hanahan and Weinberg, 2011). Gene signature or gene expression signature is a single or combined group of genes in a cell with different pattern of gene expression that occurs as a result of pathogenic condition. Many studies have been made to predict gene signatures related to cancer (van de Vijver *et al*., 2002; Allahyar and de Ridder, 2015) and produce informative genes or sub-networks by considering a predefined biological network (Babaei *et al*., 2013). Gene signature that can be applied for a broad range of cancers could be highly useful in research and clinical settings as a biomarker. The discovery of molecular signatures is proving to be a powerful tool for disease diagnosis and drug discovery.

The predictive effect of single gene biomarkers is not sufficiently specific (Zhao *et al*., 2019). When a cell becomes cancerous, the changes in the gene or protein expression patterns that causes the biological characteristics as a cancer cell occur. The cluster of genes or proteins representing these changes is called a cancer molecular signature (Nilsson *et al*., 2009; Sung *et al*., 2012). Significant clinical phenotypes resulting from these changes can be predicted by cancer molecular signature. They include the progression of diseases and the consequent increase in risk rates (Mehrabian *et al*., 2007; Hur *et al*., 2011), the response to drugs used to treat diseases and the resulting toxicity evaluation (Hines *et al*., 2010; Cohen *et al*., 2011; Xie *et al*., 2012), and the prediction of recurrence or death of diseases (Pittman *et al*., 2004; Bøvelstad *et al*., 2007).

In this review, we summarized cancer molecular signature which reported previously and cancer biomarker candidates reflecting sex disparity. In addition, we described how we identified 12 sex-biased genes and their expression patterns in liver cancer patient. More importantly, we discussed that sex-biased molecular signature *CTNNB1*^{High}, *IL6*^{High}, *RHOA*^{High} and *GLIPR1*^{Low} was correlated with overall survival (OS) in liver cancer patients with sex-dependency.

CANCER MOLECULAR SIGNATURE

Recently, many significant cancer molecular signatures have been published (Tang *et al*., 2017; Erstad *et al*., 2018; So *et al*., 2020). This is due to the development of various omics capable of analyzing a large number of samples at the same time and bioinformatics capable of analyzing a database reflecting the research results and clinical information accumulated over a long period of time.

All 1207 differentially expressed genes (DEGs) between recurrent and non-recurring samples in colon cancer patients were analyzed (Xu *et al*., 2017). Through support vector machine (SVM) analysis and verification of gene expression profiling, molecular signatures including 15 genes (*HES5*, *ZNF417*, *GLRA2*, *OR8D2*, *HOXA7*, *FABP6*, *MUSK*, *HTR6*, *GRIP2*, *KLRK1*, *VEGFA*, *AKAP12*, *RHEB*, *NCRNA00152*, *PMEPA1*) were identified as indicators that inform the prognosis and recurrence risk in colon cancer patients (Xu *et al*., 2017).

Wang *et al*. (2019) analyzed 332 DEGs between normal ovarian tissue and ovarian cancer tissue and observed the associated prognosis. Sixty-four of them were significantly correlated with the OS of ovarian cancer patients, and five genes, *IGF2*, *PEG3*, *DCN*, *LYPD1*, and *RARRES1*, were selected and screened to construct a 5-gene signature (Wang *et al*., 2019). As a result of clinical analysis, patients with low expressed 5-gene signature had significantly better OS compared to patients with the high expression (p=0.0004).

CD44-high and CD24-low cells not only express cancer stem cell-related genes, but also represent epithelial-mesenchymal transition (EMT) properties. When the relative expression of CD44 and CD24 was observed in the clinical samples of oral squamous cell carcinoma patients, CD44 expression was high in tumor tissues, but CD24 was significantly low. Therefore, CD44^{high} and CD24^{low} have high potential to be used as a molecular signature of cancer stem-like cells in oral squamous cell carcinoma (Ghuwalewala *et al*., 2016).

After neoadjuvant chemotherapy for operable gastroesophageal cancer, lymph node metastasis is known as the only proven variable that can predict the prognosis (Smyth *et al*., 2016). In the high- and low-risk groups of OS in The Medical Research Council Adjuvant Gastric Infusional Chemotherapy trial samples, 7-gene signature (*CDH1*, *ELOVL5*, *EGFR*, *PIP5K1B*, *FGF1*, *CD44v8.10*, *TBCEL*) could independently predict the patient's prognosis (Smyth *et al*., 2018). These results suggest that stratification of patients using this 7-gene signature may help in postoperative chemotherapy selection.

In hepatocellular carcinoma (HCC), there have been reported on several molecular signatures related to cancer growth and malignancy. Molecular signature including five genes, *ANGPT2*, *NETO2*, *ESM1*, *NR4A1* and *DLL4*, can be showed HCC growth, invasion into blood vessels, recurrence of cancer, and degree of intrahepatic metastasis (Villa *et al*., 2016). MicroRNA signature related to intravenous invasion and metastasis can be used to predict disease free survival and OS: highly upregulation of miR-219, miR-207 and miR-338, and extremely downregulation of miR-34, miR-30, and miR-148 (Budhu *et al*., 2008). The gene signature reflecting abnormal DNA methylation in HCC, *SCAND3*, *SGIP1* and *PI3*, can be used to determine the risk of recurrence in patients with resected early-stage HCC (Qiu *et al*., 2017). Molecular signatures in HCC can contribute to the development of targeted treatment regimens. In addition, more accurate prognosis can be predicted after treatment, selective and intensive monitoring of patients with poor prognosis can be performed, and clinical trial design can be improved, such as subdividing diseases with similar advanced stages (Erstad *et al*., 2018).

SEX-BIASED CANCER BIOMARKER CANDIDATES

Men and women show distinct sex/gender-biased differ-

ences in various areas, including growth rate and lifespan, metabolism and immune mechanisms, which are affected by carcinogenesis and cancer progression, treatment mechanisms, and survival. This is caused by sexual dimorphism that occurs due to sex-biased differences including genetic and epigenetic mechanisms as well as sex hormones that circulate in the body and induce sex disparities. Sex disparity have also been demonstrated in the expression of biomarkers that predict prognosis and diagnosis of cancer and the rate and pattern of cancer metastasis, and the response to various trials of treatment in different cancer types (Pal and Hurria, 2010; Mervic, 2012).

There are sex disparities in the epigenetic mechanisms of autosomal and sex chromosome genes (El-Maarri *et al*., 2007; Tobi *et al*., 2009; Reviewed in Yuasa, 2010). And impairment of epigenetic regulation is known as an important mechanism for the incidence and progression of cancer (Tobi *et al*., 2009). DNA methylation is the most extensively applied epigenetic marker that represented by sex-biased expression of gene such as DNA methyltransferases (DNMTs) (Nugent *et al*., 2015; Mosley *et al*., 2017). Reizel's group reported for DNA methylation in the liver, where males are hypomethylated compared to females due to testosterone exposure, and demonstrated that it was regulated by DNA methylation with sex disparity (Reizel *et al*., 2015).

One of the most striking differences of epigenetics in male and female is the inactivation of additional X-chromosomes in female cells (Rubin *et al*., 2020). Some long non-coding RNA (lncRNA), such as five prime to Xist (FTX), and a lot of epigenetic modifiers located on X chromosome, such as lysine demethylases *KDM6A* and *KDM5C*, are concerned with inactivation of X chromosome and has been known as presumed tumor suppressors in HCC (Wijchers and Festenstein, 2011; Liu *et al*., 2016). They are highly expressed in female HCC patients and suppress the proliferation and invasion of HCC cells (Xu *et al*., 2008; Liu *et al*., 2016; Snell and Turner, 2018). Their expressions correlate positively with cancer survival and decrease the risk of liver cancer in females.

Sex-biased metabolic pathways also influence cancer progression and treatment mechanisms. In the overall metabolic mechanisms, the expression of molecules involved in carbohydrate and amino acid metabolism is increased in men, and the expression of molecules involved in fatty acid metabolism is increased in women (Mittelstrass *et al*., 2011; Garcia-Herreros *et al*., 2012; Krumsiek *et al*., 2015; Ippolito *et al*., 2017). In spite of the need for higher mitochondrial activity, female mitochondria produce less reactive oxygen species (ROS), one of the causes of cell damage (Borras *et al*., 2003; Harish *et al*., 2013). Fundamental sex disparities in metabolic pathways, such as the use of nutrients in the body and mitochondrial function, can lead to sex-biased differences in the incidence and progression of cancer, and further, the mechanism of application of chemotherapy.

In general, females have a stronger and more adaptable immune response system than males (Cook *et al*., 2009; Klein and Flanagan, 2016). These immune responses with sex disparity may contribute to differences in cancer progression and mortality according to sex. Females have a greater number of neutrophils and macrophages and more active phagocytosis than males due to the inhibited secretion of inflammatory cytokines by estrogen (Scotland *et al*., 2011; Laffont *et al*., 2017). These researches have suggested that estrogen decrease the risk of cancer in females. The X chromosome contains the largest number of immune-related genes in the entire human genome such as *FOXP3* and *CD40L* (Fish, 2008; Libert *et al*., 2010; Pinheiro *et al*., 2011; Bianchi *et al*., 2012). Given the differences of expression of sex chromosome genes in male and female, X-linked immunoregulatory genes and sex hormones are expected to play an important role in mediating sex-biased immune response.

Sex hormones additionally affect sex disparity in the angiogenesis process in cancer *via* different expression of circulating angiogenic factors by intrinsically different endothelial cells (ECs) (Addis. *et al*., 2014). As shown in Evanson's study, platelet-rich plasma of adult females has more pro-angiogenic factors, including vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet-derived growth factors (PDGFs), and so on (Evanson *et al*., 2014). On the other hand, several angiogenic growth factors, such as basic fibroblast growth factor (bFGF), transforming growth factor-beta 1 (TGF-β1) and tumor necrosis factor-alpha (TNF-α), were higher expressed in male plasma (Xiong *et al*., 2018). However, there is no clear elucidation of how certain growth factors and cytokines have sex-biased expression, and how this sex disparity translates into differential angiogenesis signals and functions (Rubin *et al*., 2020). These findings suggest the need for analysis of correlation between pro- or anti-angiogenic factors and sex.

The sex-biased molecular differences induced by the various causes of sex disparity mentioned earlier were identified via systematic evaluation of omics and big data analysis. These identified molecules applied as sex-biased potential biomarker candidates (Li *et al*., 2018; Shin *et al*., 2019).

Efforts have been made to elucidate sex-biased gene expression and functions in liver cancer. Wu *et al*. (2019) suggested *CDK1*, *CCNB1*, *CYP3A4* and *SERPINA4* as sex-biased cancer molecular signature which have been identified as DEGs with sex dimorphism in HCC *via* gene expression profiling, the most frequent type of liver cancer (Wu *et al*., 2019). Phosphoglucomutase-like protein 5, encoded by the *PGM5* gene, was shown to have potential as a male-specific prognostic biomarker reflecting overall survival (OS) probability in liver cancer (Jiao *et al*., 2019). However, there is no known marker indicating the risk of liver cancer in female patients.

Although sex-biased differences in HCC development risk are well recognized (Setiawan *et al*., 2016; Wu *et al*., 2018; Yu *et al*., 2019; Rich *et al*., 2020), the prognosis between sexes remains unclear. Sex hormones are expected to play an important role in the sex disparity of malignancy. Androgen/androgen receptor signaling is known to be involved in tumor promotion as well as estrogen/estrogen receptor signaling is involved in tumor protection in mouse models (Li *et al*., 2012). Function of sex hormones in HCC has been suggested as restrain of interleukin-6 and STAT3 inactivation (Naugler *et al*., 2007; Hou *et al*., 2013). Therefore, in order to understand why male and female patients show difference in HCC development and prognosis, sex-biased molecular signature would be required to predict and evaluate the prognosis of liver cancer.

We conducted a PubMed search (https://www.ncbi.nlm.nih.gov/pubmed/) for papers describing sex-biased genes known to be related to cancer. Using five keywords, 'cancer,' 'malignancy,' 'sex,' 'gender,' and 'gene,' the PubMed search resulted in 598 related papers. After carefully checking these papers,

Table 1. List of previously reported cancer biomarker candidates with sex disparity identified by a PubMed search

Symbol	Gene name	Sex-biased function in cancer	References
BAP1	*BRCA1 Associated Protein 1*	- Regulation of cell cycle, cellular differentiation, and DNA damage - More frequent mutation in female-derived HCC	Li *et al.*, 2018; Masoomian *et al.*, 2018
BRUCE (BIRC6)	*BIR Repeat-Containing Ubiquitin-Conjugating Enzyme*	- Regulation of tumor cell death - Higher levels of expression specific in female	Salehi *et al.*, 2017
CTNNB1	*Catenin Beta 1*	- Regulation of cell growth and adhesion between cells - More frequent mutation in male liver cancer patients	Xia *et al.*, 2006; Li *et al.*, 2018
FOXA1	*Forkhead Box Protein A1*	- Regulation of apoptosis and cell cycle - Significantly expressed higher in female HCC	Li *et al.*, 2012, 2017
GLIPR1	*Glioma pathogenesis-related protein 1*	- Regulation of cell growth and chemokine secretion - Lower expressed specific in male thyroid cancer patients	Li *et al.*, 2011; Zhang *et al.*, 2015
GSTO1	*Glutathione S-transferase omega-1*	- Protection of normal cells against damage induced by carcinogens in HCC - Significantly associated with overall survival in HCC patients	Niu *et al.*, 2009; Qu *et al.*, 2015
GSTP1	*Glutathione S-transferase pi-1*		
IL6	*Interleukin 6*	- Strong correlation between inflammation and cancer - Low expression reduced risk of cancer in female liver cancer patients	Naugler *et al.*, 2007; Liu and Liu, 2014; Kumari, *et al.*, 2016
KISS1R	*Kisspeptin receptor 1*	- Suppression of cancer metastasis - Significantly expressed highly in female pituitary tumors patients	Shirasaki *et al.*, 2001; Yaron *et al.*, 2015
PER1	*Period 1*	- Regulation of cell cycle and promotion of DNA repair - Higher expression in female colon cancer patients	Wang *et al.*, 2015
RHOA	*Ras Homolog Family Member A*	- Promotion of tumor cell proliferation and metastasis - Significant reduction in survival of the entire cohort and across gender subgroups	De Rienzo *et al.*, 2016
SRPK1	*Serine-arginine protein kinase 1*	- Regulation of mRNA splicing - Higher expression significantly correlated with sex specific to male HCC patients	Graveley, 2000; Zhang *et al.*, 2016

12 cancer-related genes that showed sex-biased differences were selected for further analysis (Table 1). These genes code for proteins with critical roles in the cell cycle, cellular differentiation, regulation of cell death and growth, or cancer development processes such as angiogenesis and metastasis.

SEX/GENDER-BIASED OVERALL SURVIVAL PROBABILITY OF LIVER CANCER PATIENTS WITH SELECTED CANCER-RELATED GENES

To alleviate the aggressive progression of liver cancer in women, timely and appropriate diagnosis that reflects individual differences, such as sex/gender disparities, is essential. Identified 12 genes which showed practical relevance to cancer and sex disparities *via* a PubMed literature search were investigated the correlation between expression of these genes

and cancer malignancy by Kaplan-Meier (KM) plot analysis using clinical data from liver cancer patients in The Cancer Genome Atlas (TCGA) database. Among the 12 sex-biased genes, 7 genes (*BAP1*, *CTNNB1*, *FOXA1*, *GSTO1*, *GSTP1*, *IL6*, and *SRPK1*) showed sex-biased function in liver cancer (Table 1), suggesting that cancer biomarker candidates with sex disparity may be reliable in liver cancer.

The repositories in TCGA datasets of male and female liver cancer patients with available survival data were analyzed for the 12 genes. In order to obtain survival data for male and female liver cancer patients, the transcriptomic dataset in TCGA (version 2016_01_28; https://cancergenome.nih.gov/) was analyzed and plotted to KM plots using The Kaplan Meier Plotter (http://kmplot.com). Male (N=246) and female (N=118) liver cancer patients were divided using the auto-select best cutoff criteria. The cutoff values for high and low expression for each gene were as follows: *catenin beta 1* (*CTNNB1*, female, 7932; male, 10396), *IL6* (female, 3; male, 2), *glioma pathogenesis related 1* (*GLIPR1*, female, 153; male, 399), and *Ras Homolog Family Member A* (*RHOA*, female, 13302; male, 13613). A detailed description of the patient populations is given in Table 2. Based on TCGA data, KM plots were generated to check the correlation between expression of the 12 genes and OS probability in male and female liver cancer patients (N=246 and 118, respectively). The OS hazard ratio (HR) of the 12 genes and the statistical significance (logrank P) in the KM plots are listed in Table 3 and depicted in Fig. 1.

Table 2. The detailed description of sample populations of male and female liver cancer patients from TCGA

Cohort			RNA-seq
Platform			Illumina HiSeq 2000
Patients	Total N		364
	Sex	Male	246
		Female	118
	Race	White/Caucasian	184
		Black or African-American	17
		Asian	158
Pathology	Stage	I	171
		II	86
		III	85
		IV	5
	Vascular Invasion	None	205
		Micro	93
		Macro	16

Three out of the 12 genes, *CTNNB1*, *IL6* and *GLIPR1*, showed sex disparity in OS probability in liver cancer patients (Fig. 1A-1C). Male liver cancer patients with relatively high expression of *CTNNB1* showed a higher OS probability than did male liver cancer patients with low expression of *CTNNB1* (HR=0.67). In contrast, female liver cancer patients with relatively high expression of *CTNNB1* showed a lower OS probability and higher risk than did female liver cancer patients with low expression of *CTNNB1* (HR=1.91, logrank *p*=0.026). These data indicate that there is a positive correlation between *CTNNB1* expression and OS probability in male liver cancer patients, but there is a negative correlation in female liver cancer patients.

As shown in Fig. 1B, male liver cancer patients with relatively high expression of *IL6* showed a significantly higher OS probability than did male liver cancer patients with low expression of *IL6* (HR=0.61, logrank *p*=0.029). In females, liver cancer patients with relatively high expression of *IL6* showed a significantly lower OS probability than did patients with low expression of *IL6* (HR=2.34, logrank *p*=0.018). These results suggest a positive correlation between *IL6* expression and OS probability in male liver cancer patients, but a negative correlation in female liver cancer patients, as in the case for *CTNNB1*. These data show that female liver cancer patients with higher expression of *IL6* had poorer prognoses than those with lower levels of *IL6*.

In the analysis of *GLIPR1* (Fig. 1C), male liver cancer patients with relatively high expression of *GLIPR1* showed a

Table 3. Values of hazard ratio (HR) and specificity (logrank P) of 12 genes in male and female liver cancer patients by the KM plot analysis

Symbol	Gene name	Male		Female	
		HR (95% CI)	Logrank *p*	HR (95% CI)	Logrank *p*
BAP1	*BRCA1 Associated Protein 1*	0.77 (0.49-1.2)	0.25	1.47 (0.85-2.54)	0.17
BRUCE (BIRC6)	*BIR Repeat-Containing Ubiquitin-Conjugating Enzyme*	0.75 (0.47-1.19)	0.22	0.83 (0.48-1.45)	0.52
*CTNNB1**	*Catenin Beta 1*	0.67 (0.39-1.14)	0.14	1.91 (1.07-3.4)	0.026
FOXA1	*Forkhead Box Protein A1*	0.71 (0.44-1.15)	0.16	0.68 (0.35-1.34)	0.26
*GLIPR1**	*Glioma pathogenesis-related protein 1*	1.51 (0.97-2.36)	0.069	0.54 (0.3-0.96)	0.034
GSTO1	*Glutathione S-transferase omega-1*	0.67 (0.38-1.16)	0.15	0.76 (0.4-1.45)	0.41
GSTP1	*Glutathione S-transferase pi-1*	0.83 (0.53-1.29)	0.41	0.59 (0.32-1.1)	0.091
*IL6**	*Interleukin 6*	0.61 (0.38-0.96)	0.029	2.34 (1.13-4.83)	0.018
KISS1R	*Kisspeptin receptor 1*	1.38 (0.86-2.21)	0.17	1.68 (0.96-2.93)	0.066
PER1	*Period 1*	0.39 (0.25-0.62)	3.6e-05	0.51 (0.28-0.93)	0.025
RHOA#	*Ras Homolog Family Member A*	1.65 (1.06-2.56)	0.026	1.86 (1.06-3.27)	0.029
SRPK1	*Serine-arginine protein kinase 1*	1.74 (1.1-2.75)	0.017	2.15 (1.22-3.8)	0.0066

*Gene with a sex disparity in the correlation between expression and overall survival in liver cancer patients.

#Gene with a high HR value in both male and female liver cancer patients, but prominent sex differences with longer survival periods.

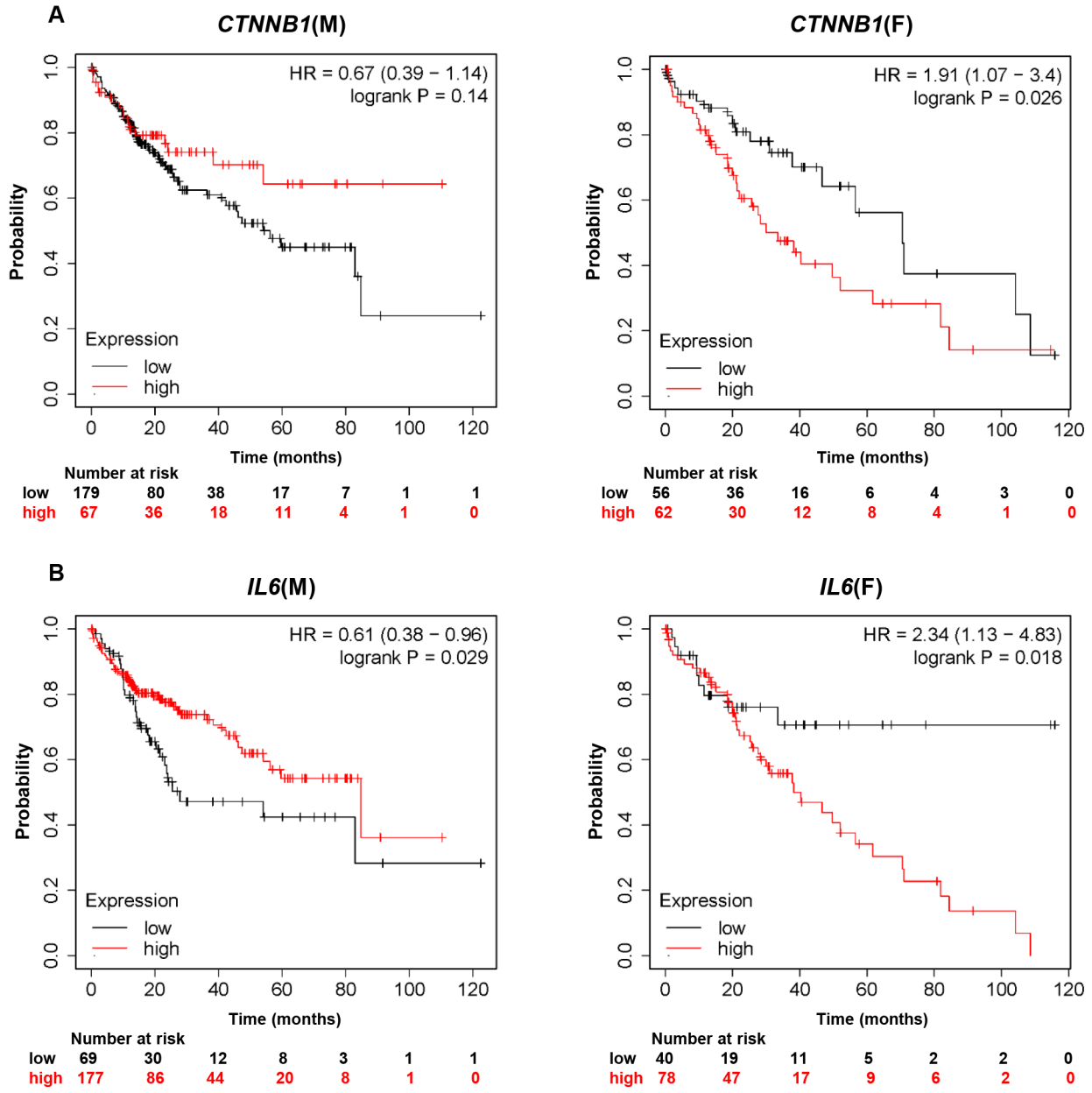

Fig. 1. Kaplan-Meier analysis assessing overall survival time according to gene expression in male and female liver cancer patients using TCGA databases. Kaplan-Meier survival curves comparing overall survival probability with expression levels of (A) *CTNNB1*, (B) *IL6*, (C) *GLIPR1*, or (D) *RHOA* in male and female liver cancer patients. *CTNNB1*, *catenin beta 1*; *IL6*, *interleukin 6*; *GLIPR1*, *glioma pathogenesis-related protein 1*; *RHOA*, *Ras homolog family member A*; HR, hazard ratio.

lower OS probability than did male liver cancer patients with low expression of *GLIPR1* (HR=1.51). Female liver cancer patients with relatively high expression of *GLIPR1* showed a significantly higher OS probability than did female liver cancer patients with low expression of *GLIPR1* (HR=0.54, logrank *p*=0.034). Unlike CTNNB1 and *IL6*, there is a negative correlation between *GLIPR1* expression and OS probability in male liver cancer patients, whereas a positive correlation exists in female liver cancer patients.

There was no apparent sex difference between males and females in HR for *RHOA* (HR=1.65 in males and HR=1.86 in females). Of note, the difference in OS probability between high *RHOA* expression and low *RHOA* expression was more drastic in male liver cancer patients than in female liver cancer patients (Fig. 1D). Taken together, our analysis demonstrates a sex-dependent correlation between OS probability in liver cancer patients and expression levels of *CTNNB1*, *IL6*, *GLIPR1*, and *RHOA*.

CTNNB1 codes for β-catenin, a known key molecule in canonical WNT signaling (Behari, 2010). The WNT/β-catenin

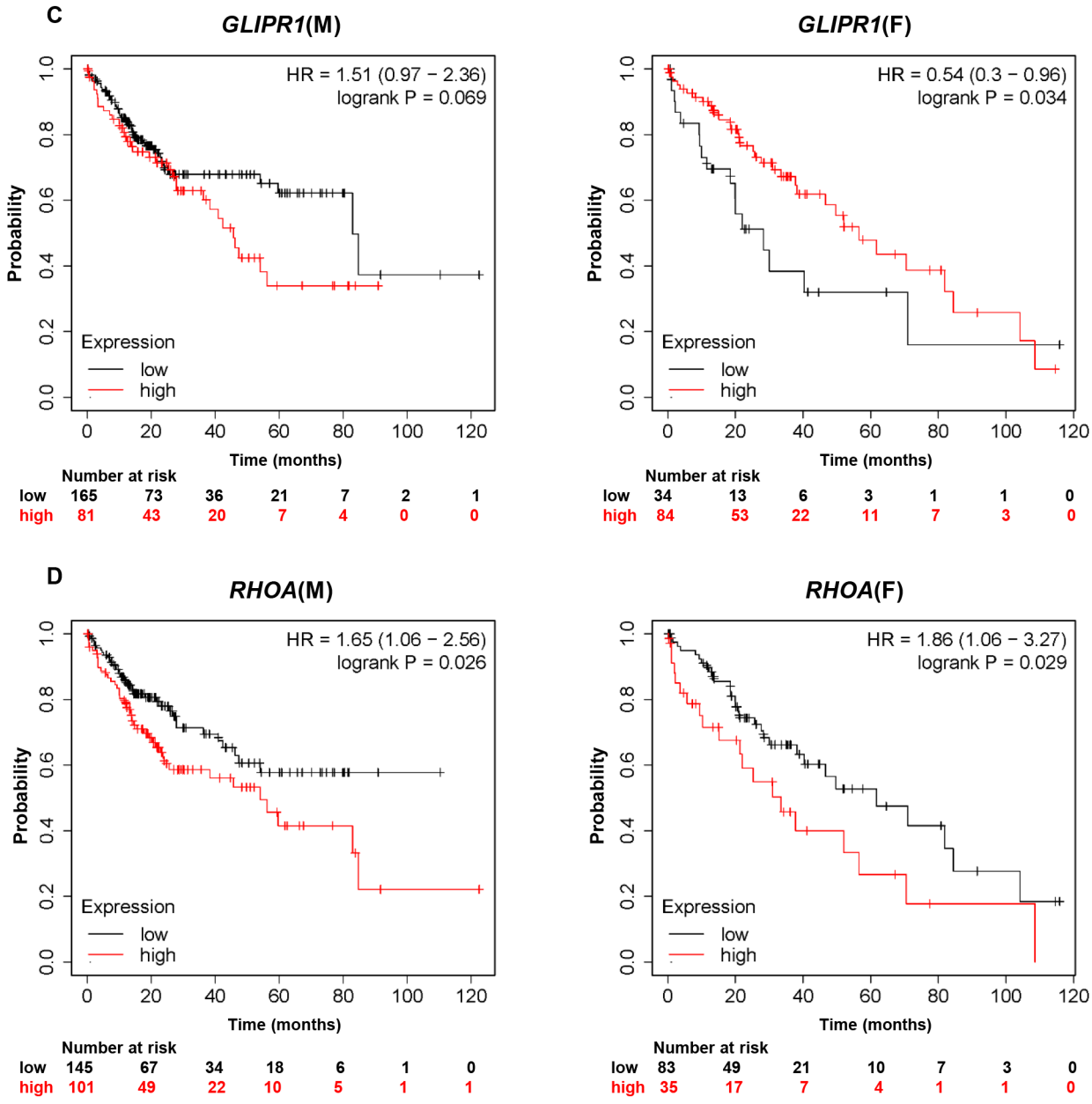

Fig. 1. Continued.

pathway is a critical regulator in cancers including liver cancer, and β-catenin plays an important role in liver regeneration (Barker, 2008; Behari, 2010; Li *et al*., 2018). *CTNNB1* mutations, which are observed more frequently in male liver cancer patients (Xia *et al*., 2006; Li *et al*., 2018), induce high β-catenin activity leading to liver cancer (Rebouissou *et al*., 2016).

IL6 is overexpressed in almost all types of cancer; its increased expression indicates a strong correlation between inflammation and cancer (Kumari *et al*., 2016). *IL6* expression is inhibited by estrogen in Kupffer cells in the liver, which may explain the lower incidence of liver cancer in women than men (Naugler *et al*., 2007; Liu and Liu, 2014). In the diethylnitrosamine-induced animal model of HCC, higher production of *IL6* was reported in males than in females (Naugler *et al*., 2007). Our results showed that the higher expression levels of *CTNNB1* and *IL6*, the lower the OS probability, and the difference in female liver cancer patients were all significant (Fig. 1A, 1B). It would be worthwhile to examine whether female liver cancer patients with high *IL6* levels have low estrogen levels.

The Rho family is involved in cellular proliferation and metastasis in cancer via reorganization of the actin cytoskeleton and regulation of related signaling (Heasman and Ridley, 2008; De Rienzo *et al*., 2016). In male malignant pleural mesothelioma patients, expression of *RHOA* was significantly higher in non-epithelioid tumors, and was associated with a significant reduction in survival (De Rienzo *et al*., 2016). In

this study, high *RHOA* expression was associated with low OS probability in both male and female liver cancer patients. When the survival period was extended to 80 months or longer, the difference in OS probability between the *RHOA*-high and *RHOA*-low expression groups was markedly greater in male than in female liver cancer patients, indicating a sex-biased effect of this gene (Fig. 1D).

The protein GLIPR1 has been shown to act as a tumor suppressor in thyroid and prostate cancers. Thyroid cancer occurs more frequently in women, but is more aggressive in men (Rahbari *et al*., 2010). In thyroid cancer, testosterone promotes cancer progression by reducing *GLIPR1* expression and cancer immune mechanisms (Zhang *et al*., 2015). In prostate cancer, GLIPR1 inhibits cancer development by destructing cytosolic β-catenin and c-Myc (Li *et al*., 2011). It would be worthwhile to further investigate the interaction between GLIPR1 and β-catenin. In glioma, GLIPR1 functions as an oncoprotein (Murphy *et al*., 1995; Ren *et al*., 2004; Awasthi *et al*., 2013). The role of GLIPR1 in liver cancer has not yet been determined. This study shows a sex-biased correlation between *GLIPR1* expression and OS in liver cancer patients: a negative correlation in males and a positive correlation in females (Fig. 1C).

In this study, correlations between sex-biased OS probability and the levels of expression of the four genes develop into a meaningful result. It can be expected that this is due to the influence of sex hormones, especially estrogen. Because the action of the female sex hormone estrogen is the main reason why liver cancer is superior to male (Bray *et al*., 2018; Jung *et al*., 2019). Of the four sex-biased genes from liver cancer through literature search and database analysis, *CTNNB1* and *IL6* have been reported to have estrogen correlation.

Crosstalk between Wnt signaling pathway, the representative signaling mechanisms related to *CTNNB1*, and estrogen

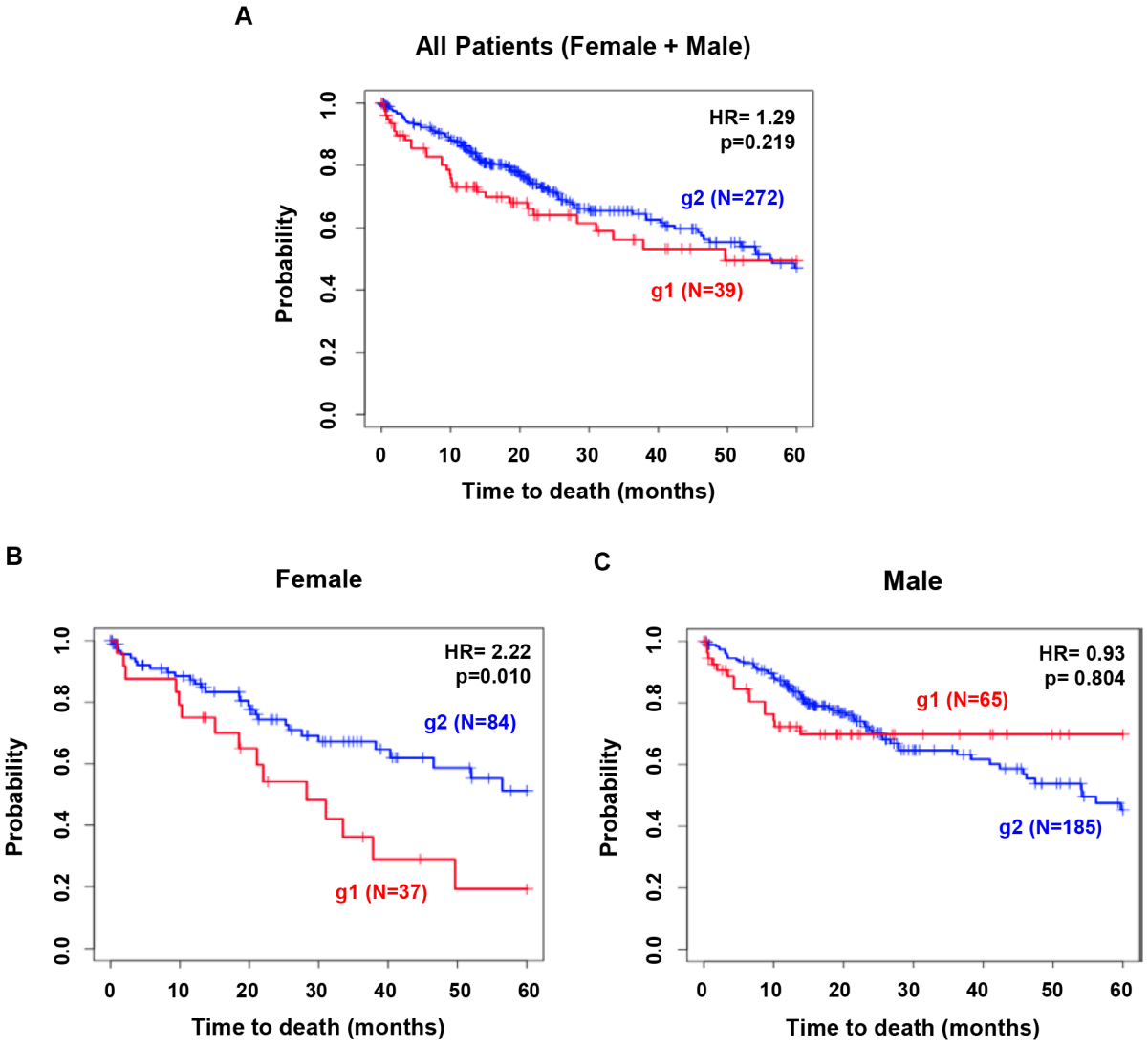

Fig. 2. Sex difference of the marker genes in predicting the clinical outcomes of liver cancer patients. Group 1 (g1), liver cancer patients with high expression of *CTNNB1*, *IL6*, and *RHOA* and low expression of *GLIPR1*; Group 2 (g2), the other patients. (A) In all liver cancer patients; (B) In female and (C) male liver cancer patients.

signaling pathway increase cell growth by inducing the transcription of cyclin D1 gene, *CCND1*, and stabilization of cyclin D1 protein (Kouzmenko *et al*., 2004; Mulligan *et al*., 2017). As mentioned earlier in the introduction, the previous studies showed that the risk of liver cancer is lower in female by suppressing IL6 secretion from Kupffer cells due to the estrogen signal (Naugler *et al*., 2007; Liu and Liu, 2014). There are also some study showing that estrogen decreases the activity of the transcription factors nuclear factor κB (NF-κB) and CCAAT/Enhancer Binding Protein (C/EBPβ), thereby reducing the promoter activity of IL6 (Stein and Yang, 1995).

There are few studies of the association between estrogen and *RHOA* or *GLIPR1* in liver cancer. According to Sailland's study, inhibition of estrogen-related receptor α (ERRα) in breast cancer increases the stability and activation of RhoA protein, which affects cell migration (Sailland *et al*., 2014). In another study, *RHOA* and sex hormones were found to have no significant relevance in the primary breast cancer (Bellizzi *et al*., 2008). There are no studies on the relationship between *GLIPR1* and estrogen. In this study, the relationship between *RHOA* or *GLIPR1* expression and sex-biased OS in liver cancer patients shows the need for further study.

SEX-BIASED MOLECULAR SIGNATURE FOR OVERALL SURVIVAL OF LIVER CANCER PATIENTS

In order to predict clinical outcomes of liver cancer in a sex-biased manner, four genes in this study were investigated further the possibility as a sex-biased molecular signature. To this end, these four genes grouped together and analyzed the TCGA database for overall survival of male and female liver cancer patients. The patients were stratified into two groups based on expression of the genes: group1 (g1) had the profile $CTNNB1^{High}$, $IL6^{High}$, $RHOA^{High}$ and $GLIPR1^{Low}$, whereas group 2 (g2) included the other patients. For each gene, high and low expression were determined by comparison with the average expression level for each gene.

As shown in Fig. 2A, KM plot analysis exhibited no significant difference in OS probability between g1 and g2 when male and female data were analyzed together. However, when data from males and females were analyzed individually, different results were obtained. In female patients (N=121), g1 exhibited worse prognostic outcomes compared to g2 (HR=2.224, p=0.010, Fig. 2B). In contrast, in male patients (N=250), there was no significant difference in OS between the two groups (HR=0.931, p=0.805, Fig. 2C). Taken together, these results suggest that high expression of *CTNNB1*, *IL6*, and *RHOA* and low expression of *GLIPR1* may serve as a prognostic biomarker for female liver cancer patients. The sex-biased molecular signature $CTNNB1^{High}$, $IL6^{High}$, $RHOA^{High}$, and $GLIPR1^{Low}$ proposed in this study may serve as an index for prediction and evaluation of OS in liver cancer, specifically in female liver cancer patients.

CONCLUSION

Sex/gender is an important biological variable that should be considered in all cancer research that aims to improve targeted therapies (Gabriele *et al*., 2016). Diseases cover both sex and gender concepts. In the present study, we demonstrated that the expression pattern of a set of genes ($CTNNB1^{High}$, $IL6^{High}$, $RHOA^{High}$, and $GLIPR1^{Low}$) was associated with poor OS probability in female liver cancer patients. The sex-biased molecular signature proposed in this study can be used to predict and evaluate the prognosis of liver cancer specifically in female patients. Further studies are needed to elucidate the molecular mechanisms underlying our findings.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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