CAST as A Potential Oncogene from Machine Searching in Gastric Cancer Infiltrated with Macrophage and Associated with Lgr5

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ABSTRACT

Background:

Gastric cancer (GC) is one of the leading malignancy diseases worldwide, especially in Asian. CAST is a potential oncogene in GC carcinogenesis process. The character of macrophage infiltration in GC microenvironment was also unaddressed.

Methods:

We first applied machine searching in gene candidate evaluation of GC. CAST expression was analyzed via the Human Protein Atlas (HPA) and Gene Expression Profiling Interactive Analysis 2 (GEPIA2) database. Protein-protein interaction (PPI) network was downloaded from STRING. We investigated the impact of CAST on clinical prognosis using Kaplan-Meier plotter. The correlations between CAST and Lgr5 and macrophage infiltration in GC was surveyed via TIMER 2.0. Finally, GeneMANIA was also used to evaluate the possible functional linkage between genes.

Results:

After machine-assisted searching, CAST expression was found signicant difference in the overall survival of GC patients. STRING revealed CAST related proteomics and transcriptomics associations, mainly about CAPN

family. Moreover, CAST significantly impacts the prognosis of GC from other datasets validation. Notably, high CAST expression was correlated with worse overall survival in GC patients (hazard ratio = 1.59; logrank P = 9.4 x 10^{-8}). CAST and Lgr5 expressions were both positively correlated with WNT 2 and WNT 2B. Among GC patients in several datasets, CAST and macrophage infiltration evaluated together showed no obvious trend toward poor clinical overall survival.

Conclusion:

CAST plays an important role in GC clinical prognosis and is associated with WNT 2/WNT 2B/Lgr5. Our study denmostrated that CAST in GC overall survival is regulated by macrophage infiltration.

Keywords: CAST, Lgr5, WNT, Gastric cancer, Machine assisted searching, macrophage

INTRODUCTION

Gastric cancer (GC) is one of the most important diseases worldwide. There are estimated to be more than 1 million newly diagnosed GC patients worldwide each year. GC is the fourth most common cancer and the second most common cause of death worldwide [1]. Globally, one in 33 men and one in 78 women will develop GC in their lifetime [2,3]. Since the diagnosis of GC is often advanced, the mortality rate is high. In 2018, 784,000 people died of GC worldwide, twice as many men as women, with East Asia, Eastern Europe, and South America being the regions of GC incidence and death [4]. Clinically, we can expect to see more cases of GC in the future due to the aging of the population. In recent years, we have even observed an increase in the incidence of GC in young people [5].

In GC genomics, approximately 10% of GC patients have familial genetic clusters, and about 1-3% of them have mutations [6]. Familial GC includes at least three major classifications: hereditary diffuse GC (HDGC), gastric adenocarcinoma and proximal gastric polyps and disease, and familial gastrointestinal cancers [7-9]. To explore the frontier of gastric carcinogenesis mechanism, recent studies had thought Lgr5 as an activator on the pathway of WNT signaling toward gastric adenocarcinoma cell proliferation. An overexpression of signature mark Lgr5 from the stem cell is derived from stomach, kidney, colon, hair follicle, and mammary gland [10]. Wu et al. found Lgr5 expression in the bottom of the normal gastric gland units, and revealed a differential expression in GC with varying differentiation. Furthermore, Lgr5 and Bmi1 were identified as the same stem cell population. CD133, CD26, CD44, and ALDH1 associated with Lgr5 may be functionally toward the GCs growth [11].

Calpastatin (CAST) is usually discovered at the plasma membrane and surrounding the nucleus [12]. CAST inhibits the calpains, which can translocate into the nucleus, and further regulate the pathway of WNT/β-catenin pathway [13]. The single CAST gene can manufacture eight or more CAST polypeptides, weighing from 17 to 85 kDa, with the function of binding to calpain molecules and Ca2+ dependent. The CAST/calpain system regulates a variety of cellular processes, involving remodeling of cytoskeletal/membrane attachments, multi-signal transduction pathways, and cell apoptosis. CAST/calpain system also participated in numerous membrane fusion events, such as neural vesicle exocytosis and platelet aggregation [14]. Previously, CAST had been reported as a possible novel marker in GC development. Liu's study results revealed that calpastatin level decreased in GCs. Furthermore, the ratio of (CAPN1 x CAPN2)/(calpastatin x CaM) was thought as a potential index for GC diagnosis [15].

In recent years, tumor-associated macrophage (TAM) is associated with the tumor microenvironment, acting as a tumor-promoting and a tumor-suppressing character [16]. TAM is categorized into the anti-tumor M1 phenotype (classically activated state) and the pro-tumor M2 phenotype (alternatively activated state), reflecting the Th1-Th2 polarization of T cells [17]. TAM works in innate host defense and kills tumor cells. Meanwhile, TAM also has a critical regulatory role in epithelial mesenchymal transition, angiogenesis, immunosuppression, and hampering the efficacy of chemotherapy [18,19].

However, the characteristics of CAST associated with immunological response of macrophage and relevance to Lgr5 were still unaddressed. We aimed to explore the possible interaction of the above-mentioned characters.

MATERIALS AND METHODS

The Cancer Genome Atlas (TCGA) Program Analysis by Using Machine Searching

The expression level of the CAST gene in various types of cancers was identified in the The Human Protein Atlas (THPA) database (https://www.proteinatlas.org/). We used the Python Selenium (Version 3.8) to automatically search the TCGA Database by entering different gene candidates, and we recorded all the candidate genes associated with the overall survival (OS) rate of GC. Then the most relevant genes were precisely selected, including CAST and WNT (P-value < 0.001).

Protein-Protein Interaction (PPI) Network from STRING

The STRING database (version 11.5) [20] is applied for searching for PPI scientists had interested in and worthy of investigation. Proteins relevant to the same topic could be linked by direct and indirect relationships and mapped to a weight network in STRING, containing 14094 organisms, 67.6 mio proteins, and > 20 bln interactions. Proteins are spotted as nodes and every two proteins is given as an edge and highlighted with a confidence score. Analogous functions among proteins will exhibit more if the confidence score is higher [21].

CAST Bioinformatics Analysis from Gene Expression Profiling Interactive Analysis 2 (GEPIA2) Datasets

We examined the mRNA level of CAST by comparing tumor and matched normal samples using the GEPIA2 database, which can provide cancer genomics data on the basis of TCGA, and the GTEx [22].

Human Protein Atlas (HPA) Appliances for Further Validation of CAST in Different Human Tissues

We used HPA, which is one of the most robust and comprehensive databases of protein and RNA in tissues and cells. HPA's goal of the Cell Atlas is to map the subcellular distribution of all human proteins over the course of a cell cycle in a canonical human cell. HPA includes over 85% of all human protein-coding genes data. Furthermore, both immunohistochemistry (IHC) scoring parameters and sub-cellular localization classifications will be purified to increase more cells types, organelles, and supply clinicians with bioinformatics about intra-organellar locations. HPA can help contribute to a deeper investigation for both basic and clinical research [23]. We used the transcriptomics and proteomics expressions to represent the character of CAST in different tumor tissues.

Survival Analysis from Kaplan-Meier (KM) Plotter

The cancer-survival information and CAST bioinformatics of the GC patients contained in the KM plotter database was extracted from the Gene Expression Omnibus (GEO), the Cancer Biomedical Informatics Grid and The Cancer Genome Atlas database. The following GC datasets were retrieved from the GEO database: GSE62254, GSE22377, GSE51105, GSE14210, GSE29272, and

GSE15459 (http://kmplot.com/analysis/index.php?p=service&cancer=gastric) [24]. We also acquired KM survival plots, in which the number of cancer patients for a specific period is compared between subgroups with different gene expression statuses. We determined the hazard ratio (HR) and 95% confidence intervals (CI) and log-rank P-values. A P-value < 0.05 was considered statistically significant.

TIMER 2.0 Database for Genes and Infiltrating Immune Cells

TIMER 2.0 database (http://timer.cistrome.org/) is a website recruiting a large amount of immune and gene bioinformatics, which can further analyze and summarize the tumor immune infiltration scores, such as neutrophil, macrophage, T cell, B cell, and NK cell. TIMER 2.0 can also analyze specific oncogene mutation groups, and genes were input for analysis of well-known oncogenic mutation in specific tumors [25-27]. The correlation between Lgr family, CAST, WNT family, and macrophage were surveyed, the data of which were adopted from the TCGA database. The results of surveyance were downloaded to show the outcome. The relationship between CAST gene and well-known immune infiltration in tumor were also analyzed through TIMER 2.0 for confirmation. A P-value < 0.05 was considered statistically significant.

Gene and Protein Networks Analysis

GeneMANIA (http://genemania.org/; accessed August 15, 2021, version 3.6.0) is a real-time multiple association network integration algorithms for predicting gene function [28]. The data could be extracted for gene-gene interactions (GGI) in our study. Regarding previous studies concerning the WNT family related to gastric cancer development, we surveyed the

relationships among WNT, CAST, and Lgr5 genes. Moreover, we analyzed the functions involving G protein-coupled receptor binding, canonical WNT signaling pathway, stem cell differentiation, and positive regulation of WNT signaling pathway for demonstration of GGI.

Statistical Analysis

The results of the KM plotter and TIMER 2.0 are shown with hazard ratio (HR) and Cox P-values from a log-rank test. We evaluated the correlation of gene expression using Spearman's correlation and statistical significance. Rho-value in determination of positive or negative correlation in protein/RNA expressions were applied.

RESULTS

CAST-Centered Network Interaction and Clustering Analysis

CAST was introduced into the STRING database to obtain the functional protein-correlation network. PPI network of these function protein expressions relevant to CAST contained 11 nodes and 40 edges, obtained with confidence scores toward CAPN2/CAPN1/CAPNS1 showing 0.999/0.999/0.986. The enriched P-value was 1.12×10^{-11}. K-means algorithm for clustering analysis in constructed network of interaction, causing three distinct numbers of interactive networks as represented in **Figure 1**.
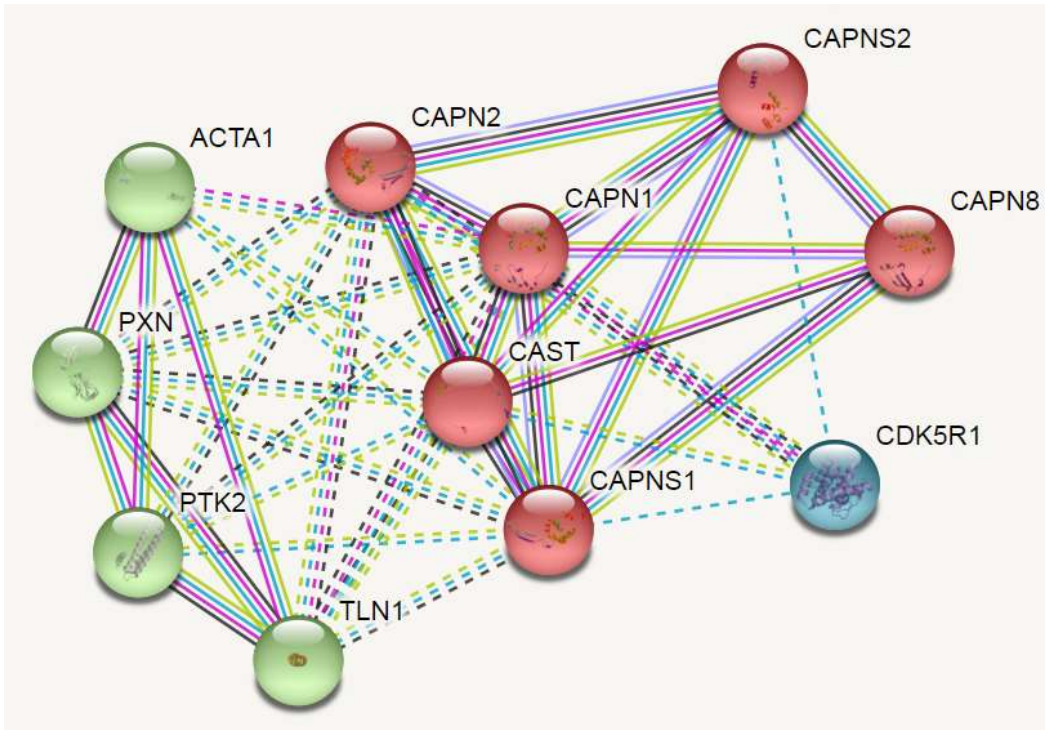
Figure 1. Important bio-targets of protein-protein interaction network in STRING clustering analysis network.

CAST Expression in Different Tissues

We extracted the CAST RNA-sequencing expression level from the GEPIA2 database. **Figure 2** demonstrated CAST expression in transcript per million (TPM). Glioblastoma (GBM), pancreatic adenocarcinoma (PAAD), and stomach adenocarcinoma (STAD) contained prominent CAST expression, while testicular germ cell tumor (TGCT), uterine corpus endometrial carcinoma (UCEC), and uterine carcinosarcoma (UCS) involved less. The certain CAST expression in different tissues was shown in **Table 1**.

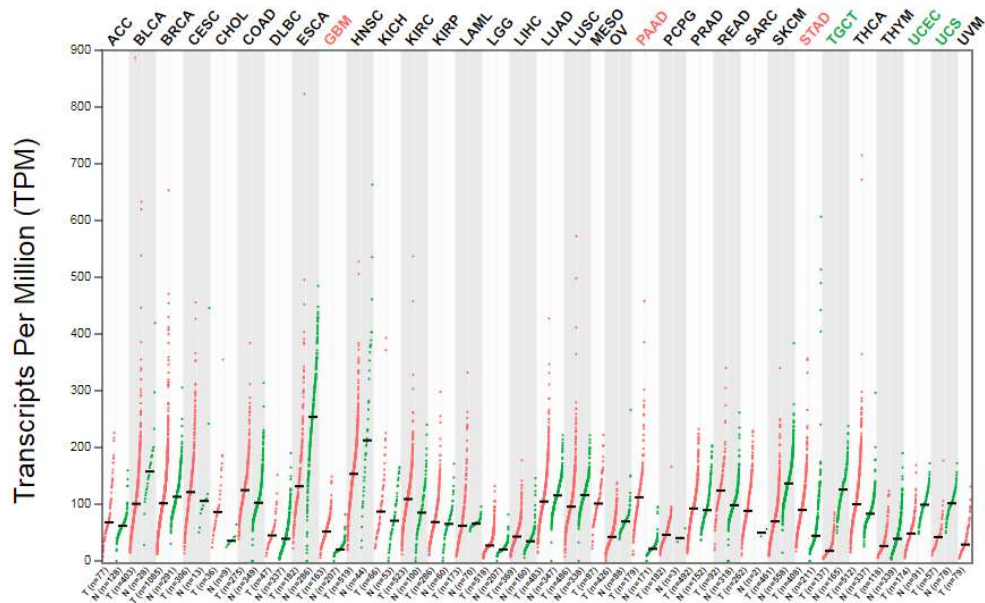**Figure 2.** Bodymap indicating CAST expression, red as tumor and green as normal.

Table 1. The median expression of CAST in different tumor and normal samples.

Site	Tumor (TPM)	Normal (TPM)
Brain	51.76	20.16
Esophagus	131.69	253.86
Thyroid	99.97	83.24
Thymus	26.31	39.02
Blood	61.86	65.73
Lung	104.7	116.04
Breast	101.62	113.17
Liver	42.91	34.63
Biliary tract	86.27	35.47
Pancreas	112.23	21.35
Stomach	89.83	44.3
Adrenal gland	67.92	61.8
Kidney	109.01	85.14
Colon	124.76	102.46
Bladder	100.67	157.63
Prostate	92.38	89.49
Testis	17.75	125.93

Validation of CAST expression in GC

To make more robust confidence in the association between CAST and GC.
We further mine the HPA revealing the cancer types are color-coded
according to which type of normal organ the cancer originates from,
including HPA036881, HPA036882, and CAB009491 from **Figure 3A, Figure
3B,** and **Figure 3C**, respectively. Zero patients with high expression, six
patients with medium expression, three patients with low expression, and
three patients with undetected expression of CAST were recorded in
HPA036881. Zero patients with high expression, two patients with medium
expression, two patients with low expression, and eight patients with
undetected expression of CAST were recorded in HPA036882. Four patients
with high expression, five patients with medium expression, one patient with
low expression, and two patients with undetected expression of CAST were
recorded in CAB009491. The RNA expression overview was displayed in
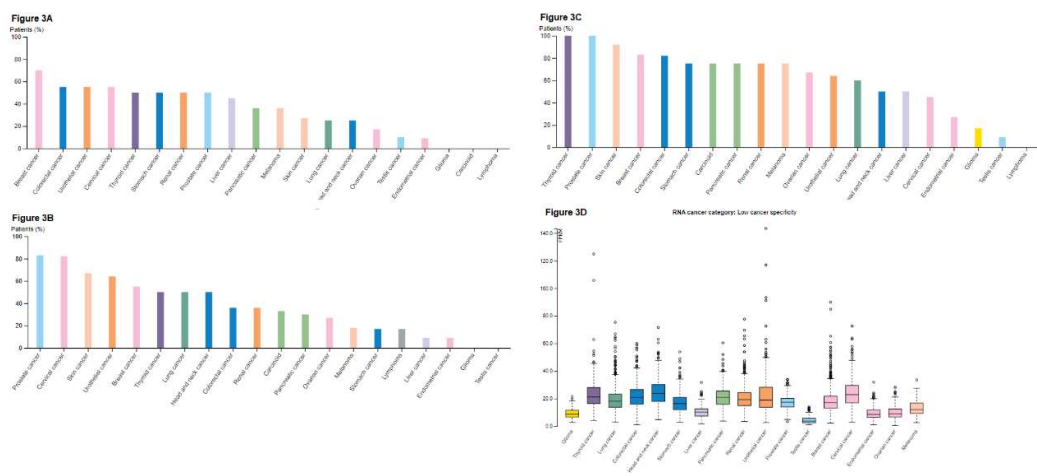**Figure 3D**.

Figure 3. (A) HPA036881 protein expression in cancer tissues showing weak to moderate cytoplasmic immunoreactivity. Few cases of basal cell carcinomas displayed strong immunoreactivity. Lymphomas along with several gliomas and testicular cancers were negative. (B) HPA036882 protein expression in cancer tissues displaying weak to moderate cytoplasmic staining with membranous positivity in several cases. Few cases of colorectal, breast, lung, skin and urothelial cancers were strongly stained. Most cases of gliomas, testicular, liver and endometrial cancers were negative. (C) CAB009491 protein expression in most cancer tissues demonstrating moderate to strong cytoplasmic positivity. Gliomas and lymphomas generally showed weak positivity while testicular cancers in most cases were negative. (D) RNA-sequencing data of cancer category from the TCGA.

CAST Associated with Survival in Gastric Cancer

Table 2 showed significant survival difference between low expression and high expression cohorts (All, GSE22377, GSE14210, GSE29272, GSE15459).

Table 2. CAST RNA-sequencing expression and survival analysis in gastric cancer.

Source	Median survival		FDR	P-value
	Low expression cohort (months)	High expression cohort (months)		
All	44.57	21.93	1%	<0.0001
GSE62254	18.27	22.83	100%	0.2373
GSE22377	36.4	17.2	50%	0.0297
GSE51105	39.2	20.1	>50%	0.449
GSE14210	15.9	7.9	10%	0.0024
GSE29272	32.6	18.6	>50%	0.0289
GSE15459	45.1	22.8	>50%	0.0444

In GC cohorts analyses from KM plotter, CAST was significantly related to patient survival [All, HR: 1.59, 95% confidence interval (CI): 1.34-1.88, log-rank P-value: 9.4×10^{-8}] when the median expression of CAST was set as a cutoff point to stratify patients (Figure 4A and 4B).
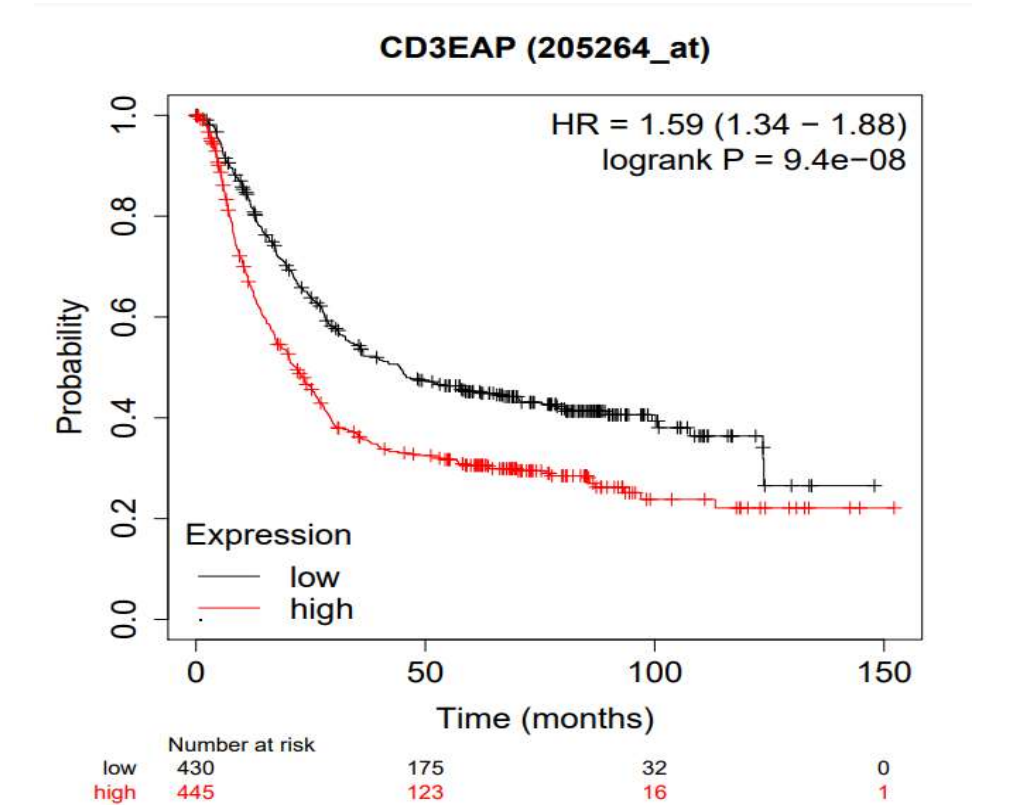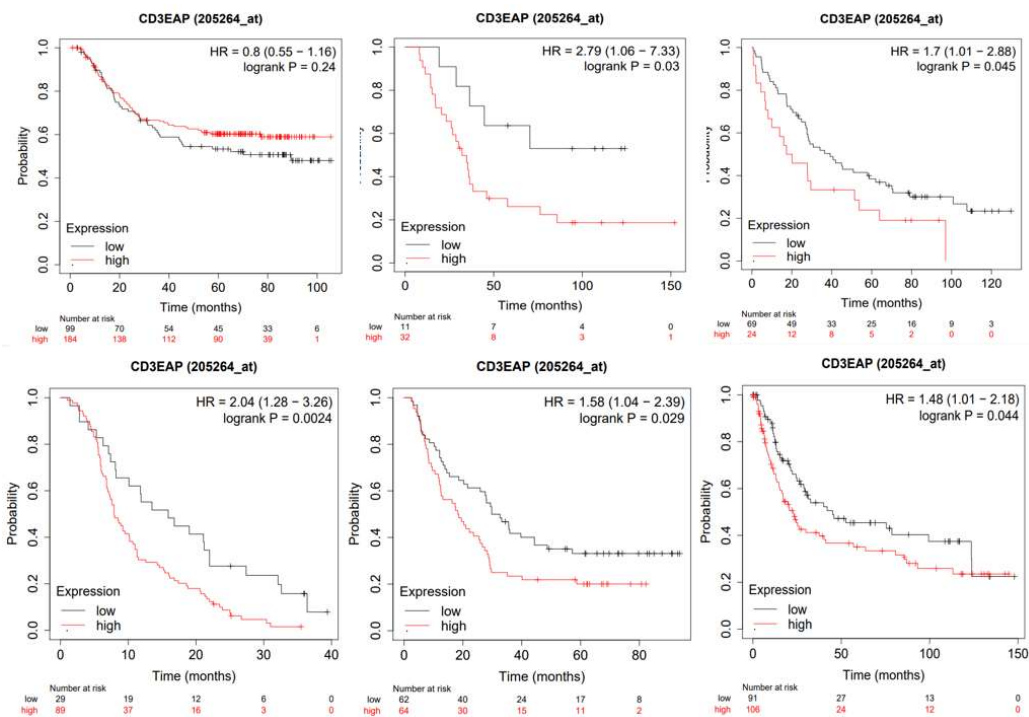
Figure 4A. All.

Figure 4B. Subgroup survival analyses.

CAST/WNT/Lgr5 Co-Expressions in Gastric Cancer

Figure 5 revealed the solitary CAST relevant to significant GC survival analysis (HR), clinical outcome (HR: 1.22; p=0.0415) of which was compatible with the dataset retrieved from KM plotter. **Figure 6** examined the correlation between CAST and WNT family. Among them, WNT2 (rho=0.128; P-value=0.009)/WNT16(rho=0.102; P-value=0.037)/WNT2B(rho=0.288; P-value<0.001)/WNT5A(rho=0.237; P-value<0.001)/WNT9A(rho=0.222; P-value<0.001)/WNT9B(rho=0.182; P-value<0.001) showed positively significant correlation. On the other hand, WNT6(rho=-0.122; P-value=0.019)/WNT3A(rho=-0.121; P-value=0.013)/WNT8B(rho=-0.141; P-value=0.004) showed negatively significant correlation. In **Figure 7**, there were WNT2(rho=0.139; P-value=0.004)/WNT3(rho=0.2; P-value<0.001)/WNT11(rho=0.336; P-value<0.001)/WNT2B(rho=0.144; P-value=0.003)/WNT7B(rho=0.186; P-value<0.001)/WNT8B(rho=0.168; P-value<0.001)/WNT10B(rho=0.183; P-value<0.001) displaying the positively significant correlation toward Lgr5. The overlapped WNT family genes in both Lgr5 and CAST were WNT2 and WNT2B.

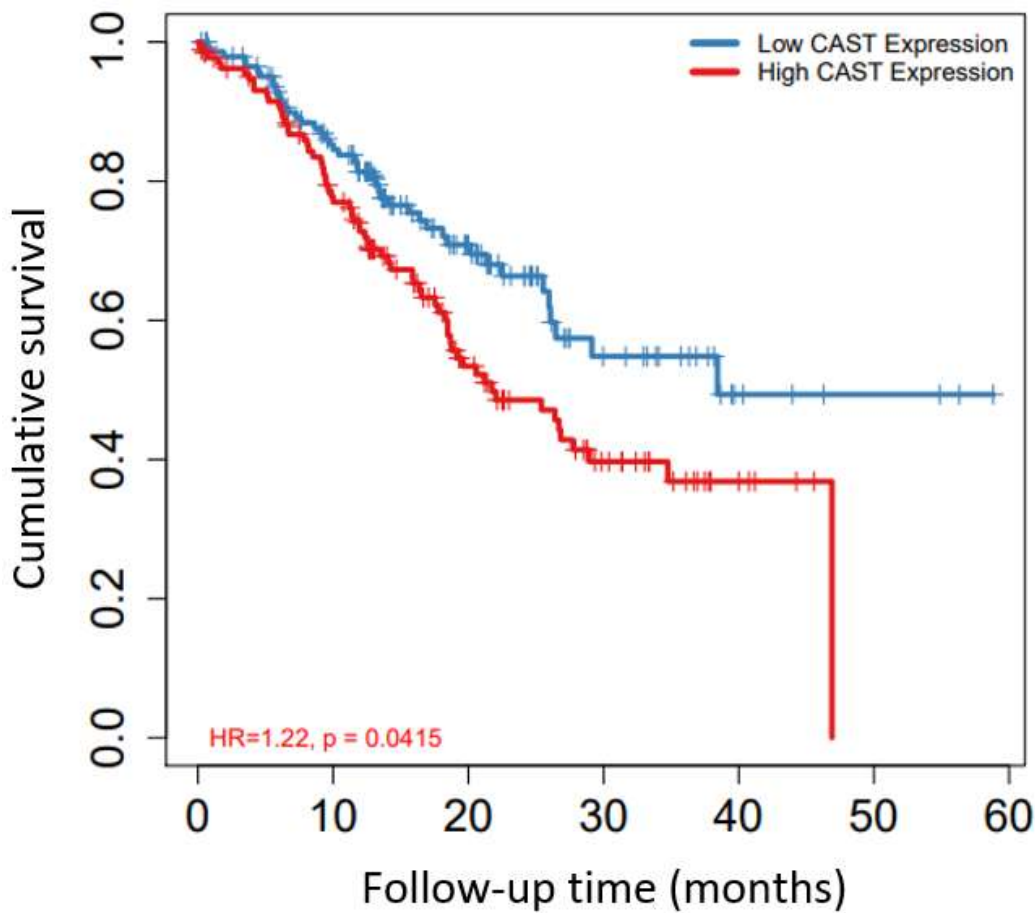**Figure 5.** Cumulative survival of different CAST expression in gastric cancers.
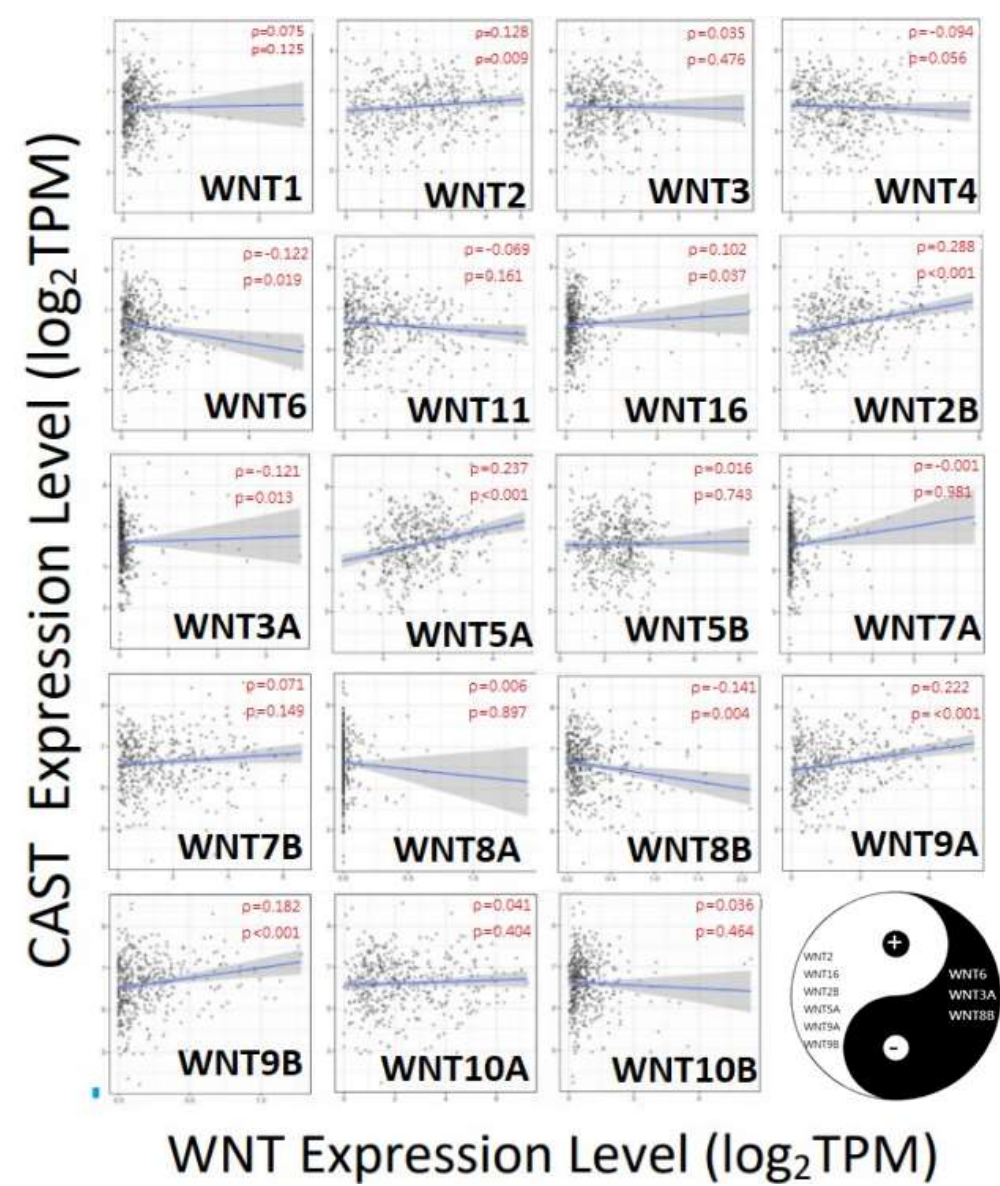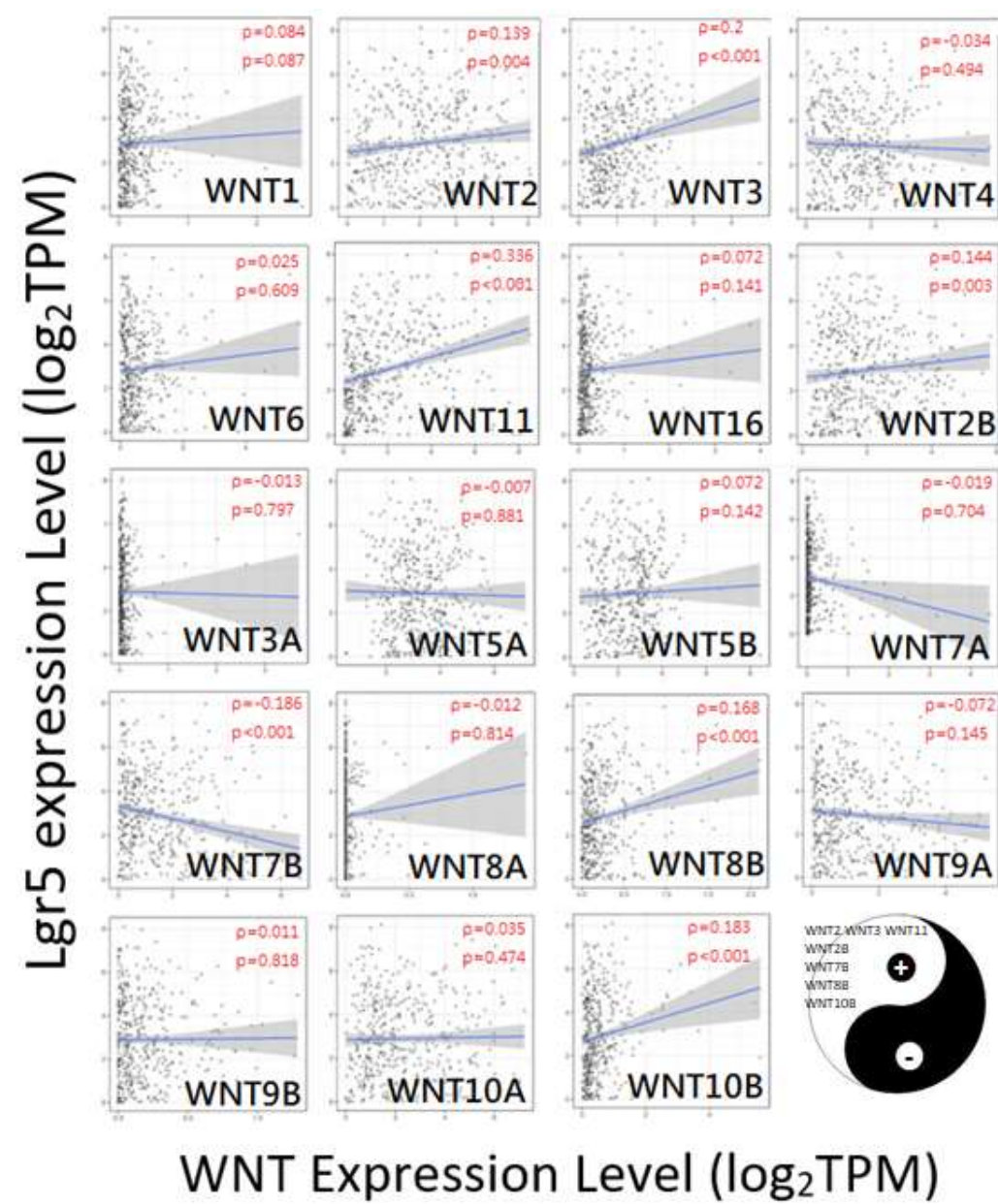
Figure 6. Correlation between CAST and WNT family.

+: Positively significant correlation.

-: Negatively significant correlation.

**Figure 7.** Correlation between Lgr5 and WNT family.

+: Positively significant correlation.

-: Negatively significant correlation.

CAST and Macrophage in Gastric Cancer

TIMER 2.0 showed databases including TIMER, EPIC, XCELL, CIBERSORT-ABS, and QUANTISEQ. We discovered that in **Figure 8**, TIMER showing high CAST expression (>50 percent) and high macrophage infiltration (>50 percent) had a significantly lower cumulative survival than high CAST expression and low macrophage infiltration (<50 percent), with a HR=2.08 and a P-value=0.00927. However, there was no significance of cumulative survival in EPIC and XCELL CAST expression and macrophage infiltration analyses. In macrophage M1 and M2 evaluations, there was no significant cumulative survival difference, either.

Figure 8. Cumulative survival in gastric cancer patients with CAST expression and Macrophage infiltration.
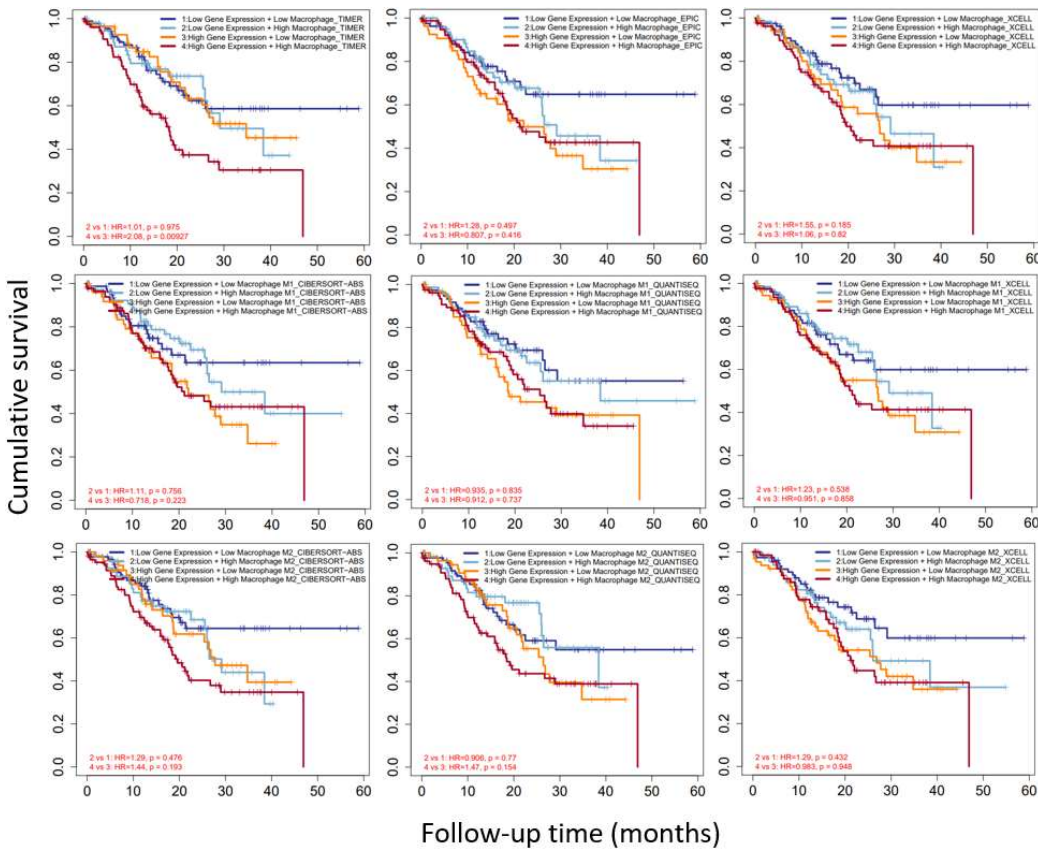
CAST-WNT2/WNT2B-Lgr5 Linkages toward Gastric Carcinogenesis

We input CAST, WNT2, WNT2B, and Lgr5, using GeneMANIA and found that CAST was linked to WNT family and Lgr family as shown in **Figure 9**. WNT2 and WNT2B contained G protein-coupled receptor binding. WNT2 had a canonical WNT signaling pathway, but WNT2B didn't involve it. Lgr5 recruited positive regulation of WNT signaling pathway and canonical WNT signaling pathway.

Figure 9. Interactions among CAST, WNT2/WNT2B, and Lgr5
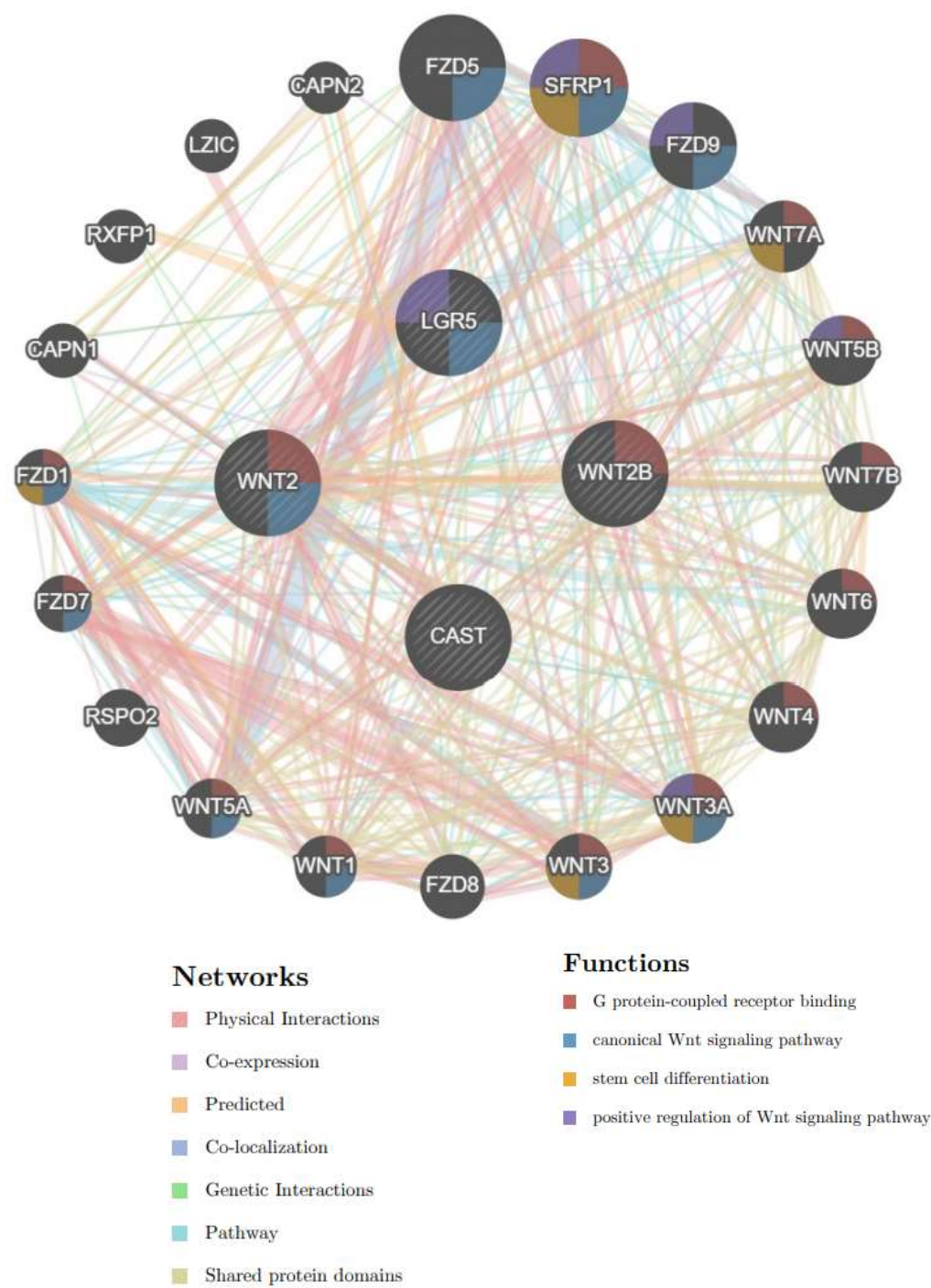
DISCUSSION

In our present study, we demonstrated that CAST plays as an oncogene associated with Lgr5 in gastric cancer via the WNT signaling pathway. WNT2 and WNT2B expressions were found significantly positive correlation with both CAST and Lgr5, which left a further study orientation for GC molecule biochemistry, transcriptomics, and proteomics. Though CAST is discovered a prominent impact on GC patients' survival after multivariate adjustments, multi-databases datasets revealed that macrophage might be a key role in immune regulation in GC microenvironment toward tumor suppression.

Our research revealed CAST as a potential oncogene toward GC formation. Previous studies seldom focused on this novel issue. Liu et al. [15] appointed that except for CAST, CAPN1, CAPN2, and CaM might also contribute to GC formation, which is partially compatible with our results. The Calpain system was also associated with colorectal adenocarcinoma and prostate cancer, which suggested that calpains might be an important character in tumorigenesis tumor progression [29,30]. Calpain system is relevant to human epidernal growth factor receptor 2 and E-cadherin in breast cancer [31,32]. Meanwhile, calpain-2 was proved to contribute to the promoter methylation of CRMP4 to repress the transcription, heading to the metastasis of prostate cancer by enhancing vascular endothelial growth factor C expression [33].

The mechanism of CAST resulting GC was still unclear. We tried to find the relevant gene expression or possible pathways. After databases mining, Lgr5 and CAST might regulate the GC formation via the same pathway-WNT family, especially WNT 2 and WNT 2B, as our novel findings in the GC formation signature. WNT/β-catenin pathway in gastric cancer showed an

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important feature in regulating proliferation, stem cell maintenance, and homeostasis in gastric mucosa [34,35]. More than 30% of GC in which activated WNT/β-catenin signaling can be found. The fundamental role of WNT/β-catenin signaling in the self-renewal of GC stem cells has been demonstrated [36-38]. WNT/β-catenin signaling paradox was also an issue recently, discussing WNT signaling hyperactivation by mutations in β-catenin destruction complex components or β-catenin itself contributes to tumorigenesis [39]. β-catenin can be further activated by additional layers of regulation, which represented as the complicated role of WNT signaling deregulation in cancer [40-42]. The double-sided function (tumorigenesis or tumor suppression) of WNT/β-catenin system was disclosed in our clinicopathological datasets survival follow-up.

Recently, TAM was discovered to be associated with WNT signaling in tumor microenvironment. Wu et al. [43] demonstrated that macrophages play a protumorigenic role in GC patients. The possible mechanism might be originated from tumor microenvironment related inflammation, matrix remodeling, angiogenesis, seeding at distant sites, intravasation, and tumor cell invasion [44]. The current studies also give scientists a clue that macrophages may play a helpful or harmful role in GC microenvironment. Huang et al. also demonstrated that the heterogeneity of macrophages within the tumor is present at both macro- and micro-levels due to the gradient change of different markers [45]. In our study, the role macrophage infiltration in GC associated with CAST revealed the unsure character in GC formation and survival. We hypothesize that macrophage infiltration could manipulate the exact signaling pathway of GC carcinogenesis process. Maybe further in vitro researches should be launched to unblind the mechanism.

We had confidence in database mining for genes and macrophage relevant to GC on the basis of some characteristics such as high reproducibility, high convenience, and no need of inform and consent from patients. The analytic methodology of our article is very suitable to establish a precise/personalized evaluation of a molecular investigation of GC. Though we found a novel marker and immune infiltration correlated with GC, we had acknowledged some limitations in our study. First, though databases contained a lot of bioinformatics online, we still need to explore further experiments for external validation for the results. Second, the detailed mechanism of how these genes (CAST, WNT, and Lgr5) cause the carcinogenesis of GC was still a question mark. However, we could use databases to make preliminary reports of these genes, to facilitate the confidence of future novel GC carcinogenetic models. Third, we need tissue sample confirmation due to the possible potential tumor purification error.

CONCLUSION

Our study explored that CAST was a signature oncogene in GCs. Moreover, the CAST gene in gastric carcinogenesis was regulated by macrophage in our OS analyses. The detailed mechanism of CAST gene related GCs formation was still investigated, probably associated with Lgr5-related pathways and WNT/β catenin cellular signaling.

Data Availability Statement

The original contributions presented in the study are included in the article. Further inquiries can be directed to the first author (K.-T.Y.) and the corresponding author (J.-S.C.)

Abbreviations: GC: gastric cancer; OS: overall survival; TPM: transcripts per million; FPKM: frgments per kilobase per million; FDR, false discovery rate; ACC: Adrenocortical Carcinoma; BLCA: Bladder Urothelial Carcinoma; BRCA: Breast Invasive Carcinoma; CESC: Cervical and Endocervical Cancer; CHOL: Cholangiocarcinoma; COAD: Colon Adenocarcinoma; DLBC: diffuse Large B-cell Lymphoma; ESCA: Esophageal Carcinoma; GBM: Glioblastoma Multiforme; HNSC: Head and Neck Cancer; KICH: Kidney Chromophobe; KIRC: Kidney Renal Clear Cell Carcinoma; KIRP: Kidney Renal Papillary Cell Carcinoma; LAML: Acute Myeloid Leukemia; LGG: Lower Grade Glioma; LIHC: Liver Hepatocellular Carcinoma; LUAD: Lung Adenocarcinoma; LUSC: Lung Squamous Cell Carcinoma; MESO: Mesothelioma; OV: Ovarian Serous Cystadenocarcinoma; PAAD: Pancreatic Adenocarcinoma; PCPG: Pheochromocytoma and Paraganglioma; PRAD: Prostate Adenocarcinoma; READ: Rectum Adenocarcinoma; SARC: Sarcoma; SKCM: Skin Cutaneous Melanoma; STAD: Stomach Adenocarcinoma; TGCT: Testicular Germ Cell Tumors; THCA: Thyroid Carcinoma; THYM: Thymoma; UCEC: Uterine Corpus Endometrial Carcinoma; UCS: Uterine Carsinosarcoma; UVM: Uveal Melanoma

Author contributions

K.-T.Y., C.-J.L., R.C., and Y.-W.T. designed the study concept and the structure of the article. K.-T.Y. wrote the draft. J.-T. Wang drew the graphical abstract. J.-S.C. modified the whole article. All authors contributed to the article and approved the submitted version.

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Conflict of Interest

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

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