October 28, 2020

Dear Editors,

Please find our attached manuscript entitled "**Evaluation for the Genetic Association Between Store-Operated Calcium Influx Pathway (STIM1 and ORAI1) and Human Hepatocellular Carcinoma in Patients with Chronic Hepatitis B Infection**," which we are submitting for consideration for publication as an Original Research article in ***Biology*** **(biology-947101).** We are thankful for your kind suggestions regarding our manuscript. Here, we are sending our revised manuscript in accordance with the comments given by the two reviewers. We have read through all the reviewers' suggestions very carefully, and made the necessary revisions based on these comments, as detailed below in a point-by-point format. The revised sections are highlighted in red. Finally, we would like to thank you once again for giving us the opportunity to improve our manuscript. We very much hope that these revisions are adequate. We appreciate your assistance and are looking forward to hearing from you.

Sincerely yours,

Wei-Chiao Chang (D.Phil.; Oxon)
Professor, Department of Clinical Pharmacy,
Taipei Medical University, Taiwan
250 Wu-Hsing Street, Taipei 110, Taiwan

Reviewer 1.

Comments and Suggestions for Authors

Irham et al conducted large-scale profiling for STIM1 and ORAI1 SNP in large HBV patient sets. Though the statistical significance are low for the SNP probably due to not enough HCC patients, the data are still quite valuable to the community and have potential clinical benefits. I would happily accept the paper if the author can address my following questions.

Q1: Reviewer #1. I am quite interested in whether there are gender differences in SNP profile. Namely, since you have a large dataset, could you evaluate whether there is any SNP signature that is more dominant in males or females? And after the gender grouping, if there are any SNPs that showed significant association?

A1: We sincerely thank your valuable suggestions. It is a very important point. As suggested by reviewer, we further analyzed all the SNPs (40 SNPs) with stratified grouping by gender (male and female). Results showed that two *STIM1* SNPs (rs2959081 and rs7116520) associated with HCC progression in female, while *ORAI1* SNP rs6486795 was found in male population. However, after correction for multiple testing, none of the SNPs reached a significant level. The data was shown as below :

Associations of *STIM1* and *ORAI1* genes with gender (Male and Female)

			Male Gender N=2196 (60.48%)		Female Gender N=1435 (39.52%)	
SNP	Gene	Effect allele	(Genotype) *p* value[a] [q value]	(Dominant) *p* value[a] [q value]	(Genotype) *p* value[a] [q value]	(Dominant) *p* value[a] [q value]
rs4243966	*STIM1*	C	0.879 [0.963]	0.993 [0.993]	0.349 [0.971]	0.321 [0.975]
rs7943201	*STIM1*	A	0.776 [0.963]	0.485 [0.993]	0.417 [0.971]	0.323 [0.975]
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rs10835262	*STIM1*	A	0.913 [0.963]	0.908 [0.993]	0.920 [0.971]	0.828 [0.975]

rs4622250	*STIM1*	T	0.512 [0.963]	0.926 [0.993]	0.970 [0.971]	0.873 [0.975]
rs75197750	*STIM1*	T	0.196 [0.963]	0.131 [0.993]	0.668 [0.971]	0.380 [0.975]
rs10500589	*STIM1*	C	0.603 [0.963]	0.319 [0.993]	0.966 [0.971]	0.883 [0.975]
rs11030209	*STIM1*	C	0.642 [0.963]	0.926 [0.993]	0.905 [0.971]	0.761 [0.975]
rs10835270	*STIM1*	T	0.394 [0.963]	0.180 [0.993]	0.794 [0.971]	0.837 [0.975]
rs11030210	*STIM1*	T	0.729 [0.963]	0.945 [0.993]	0.381 [0.971]	0.479 [0.975]
rs7929653	*STIM1*	A	0.693 [0.963]	0.963 [0.993]	0.923 [0.971]	0.928 [0.975]
rs6578418	*STIM1*	G	0.240 [0.963]	0.095 [0.993]	0.328 [0.971]	0.249 [0.975]
rs10835272	*STIM1*	A	0.375 [0.963]	0.838 [0.993]	0.598 [0.971]	0.826 [0.975]
rs10742189	*STIM1*	C	0.808 [0.963]	0.755 [0.993]	0.404 [0.971]	0.232 [0.975]
rs7129444	*STIM1*	C	0.824 [0.963]	0.563 [0.993]	0.736 [0.971]	0.843 [0.975]
rs7118422	*STIM1*	T	0.926 [0.963]	0.929 [0.993]	0.715 [0.971]	0.419 [0.975]
rs4910863	*STIM1*	C	0.887 [0.963]	0.835 [0.993]	0.208 [0.971]	0.076 [0.975]
rs11030264	*STIM1*	G	0.757 [0.963]	0.476 [0.993]	0.183 [0.971]	0.068 [0.975]
rs7924984	*STIM1*	A	0.389 [0.963]	0.506 [0.993]	0.240 [0.971]	0.319 [0.975]
rs2412338	*STIM1*	T	0.396 [0.963]	0.501 [0.993]	0.163 [0.971]	0.358 [0.975]
rs10835402	*STIM1*	C	0.963 [0.963]	0.790 [0.993]	0.109 [0.971]	0.170 [0.975]
rs11030472	*STIM1*	G	0.147 [0.963]	0.078 [0.993]	0.456 [0.971]	0.214 [0.975]
rs11030478	*STIM1*	A	0.688 [0.963]	0.934 [0.993]	0.920 [0.971]	0.824 [0.975]
rs11030486	*STIM1*	T	0.698 [0.963]	0.874 [0.993]	0.926 [0.971]	0.823 [0.975]
rs12284835	*STIM1*	A	0.071 [0.963]	0.109 [0.993]	0.822 [0.971]	0.636 [0.975]
rs727152	*STIM1*	G	0.519 [0.963]	0.315 [0.993]	0.725 [0.971]	0.951 [0.975]
rs2959081	*STIM1*	C	0.608 [0.963]	0.543 [0.993]	**0.021** [0.971]	**0.005** [0.975]

rs11030639	*STIM1*	G	0.770 [0.963]	0.666 [0.993]	0.054 [0.971]	0.195 [0.975]
rs7116520	*STIM1*	G	0.410 [0.963]	0.209 [0.993]	**0.007** [0.971]	**0.001** [0.975]
rs1442725	*STIM1*	T	0.304 [0.963]	0.373 [0.993]	0.706 [0.971]	0.467 [0.975]
rs11030841	*STIM1*	A	0.611 [0.963]	0.486 [0.993]	0.381 [0.971]	0.885 [0.975]
rs4910882	*STIM1*	A	0.637 [0.963]	0.501 [0.993]	0.362 [0.971]	0.975 [0.975]
rs6486795	*ORAI1*	C	0.078 [0.963]	**0.037** [0.993]	0.971 [0.971]	0.850 [0.975]
rs74936888	*ORAI1*	C	0.304 [0.963]	0.124 [0.993]	0.419 [0.971]	0.418 [0.975]
rs3741595	*ORAI1*	C	0.184 [0.963]	0.081 [0.993]	0.731 [0.971]	0.637 [0.975]

[a] Adjusted by alanine transaminase and age. Significant *p* values are in **bold***. The q value is the false discovery rate (FDR) estimation for multi-testing.

Q2: Reviewer #1. For all the SNPs you identified, could you please show some examples that some of the SNPs can cause functional alterations to the proteins (mutations in critical domains that may cause a structural change of the protein), especially in the SNPs that shows trends to be associated with the HCC?

A2: We sincerely thank to the reviewer's comments. According to reviewer suggestions, we tried to identify the effects of the alteration/ deleteriousness of variants in protein by using some of the functional annotation tools such as the scale-invariant feature transform (SIFT), Polymorphism Phenotyping v2 (PlyPhen-2), ClinVar and Combined Annotation Dependent Depletion (CADD) databases. As shown in the following Table, four SNPs (*STIM1* rs6578418, rs11030472, rs7116520, and *ORAI1* rs6486795) are intron variants. None information was available in the three databases (SIFT, PlyPhen-2, and ClinVar). Regarding to the database of CADD, the score displayed that four variants with score less than 30 which indicated the four variants more likely to be benign.

Information about the variants consequence from various databases

SNP	Position (hg38) (bp)	Effect Allele	Consequence Type	PolyPhen2	SIFT	ClinVar	CADD
rs6578418	Chr11:3905002	G	Intron variant	NA	NA	NA	4.363(Benign)
rs11030472	Chr11:3978105	G	Intron variant	NA	NA	NA	8.781(Benign)
rs7116520	Chr11:4032147	G	Intron variant	NA	NA	NA	0.541(Benign)
rs6486795	Chr12:121638011	C	Intron variant	NA	NA	NA	0.481(Benign)

NA: Not Available

Additionally, we elaborated the relationship between the *STIM1* rs6578418, rs11030472, rs7116520 and *ORAI1* rs6486795 and gene expression. Here, we utilized the publicly available databases GTEx portal (http:// www.gtexportal.org/home/) to confirm the tissue expression quantitative trait loci. For *STIM1* SNPs rs6578418 and rs11030472, none of information is available in the GTEx portal database due to limited available tissue (Table S3). While GG genotypes of *STIM1* rs7116520 showed a higher expression in a variety of tissues including heart-left ventricle and nerve tibial lung tissue (Figure S3). Furthermore, *ORAI1* rs6486795 with CC genotypes also correlated with a higher expression compared to those with TC and TT genotypes in variety of tissues (*e.g.* whole blood, esophagus, thyroid, heart, stomach, pancreas, muscle, colon). The results have been added in the revised manuscripts[Page 13, lines 295-305] [Page 5, lines 136-139]. Furthermore, the Table (Table S3) and Figure (Figure S3) were added in the supplementary materials [Page 15, lines, 406-407 and page 16, lines 412-413]. In addition, to understand the role of store-operated calcium pathways (STIM1 and ORAI1) in HCC progression, functional studies by both total internal reflection fluorescence microscopy and transwell migration assay were applied. As shown in the Figure 2, we found that the colocolization of STIM1 and Orai1 was inhibited by 2-APB (SOC inhibitor). We further analyzed the effects of store-operated calcium pathways on cell migration in Huh 7 and HepG2 cell lines. The results showed that the cellular migration (Huh 7 and HepG2) significantly decreased by 2-APB (Figure 2B and 2C). Data has been added in revised manuscript [page 13, lines 307-325] [Pages 5-6, lines 141-171]. In addition, we added sentences in the part of discussion [Pages 15, lines 385-386] and conclusion [Pages 15-16, lines 394-399].

Table S3. Expression Quantitative trail loci (eQTL) results of The SNP from Genotype-tissue expression (GTEx).

SNP ID	Gencode ID (ENSG00000-)	Gene symbol	p value	Effect size	Tissue	Actions
rs7116520	167323.11	*STIM1*	1.1e-9	-0.18	Nerve - Tibial	GG>AG>AA
	167323.11	*STIM1*	2.0e-8	-0.22	Heart-Left Ventricle	GG>AG>AA
rs6578418	NA	NA	NA	NA	NA	NA
rs11030472	NA	NA	NA	NA	NA	NA
rs6486795	276045.2	*ORAI1*	1.30E-17	0.22	Whole Blood	CC>CT>TT
	276045.2	*ORAI1*	9.90E-16	0.29	Esophagus - Mucosa	CC>CT>TT
	276045.2	*ORAI1*	3.30E-13	0.23	Thyroid	CC>CT>TT
	276045.2	*ORAI1*	7.60E-08	0.24	Stomach	CC>CT>TT
	276045.2	*ORAI1*	4.50E-07	0.25	Pancreas	CC>CT>TT
	276045.2	*ORAI1*	4.80E-07	0.11	Muscle - Skeletal	CC>CT>TT
	276045.2	*ORAI1*	1.5E-06	0.21	Colon - Transverse	CC>CT>TT
	276045.2	*ORAI1*	0.000029	0.19	Esophagus - Gastroesophageal Junction	CC>CT>TT
	276045.2	*ORAI1*	0.000058	0.17	Heart - Atrial Appendage	CC>CT>TT
	276045.2	*ORAI1*	0.00016	0.13	Esophagus - Muscularis	CC>CT>TT

Source: Expression Quantitative trail loci (eQTL) obtained from https://gtexportal.org/home, NA: Not Available

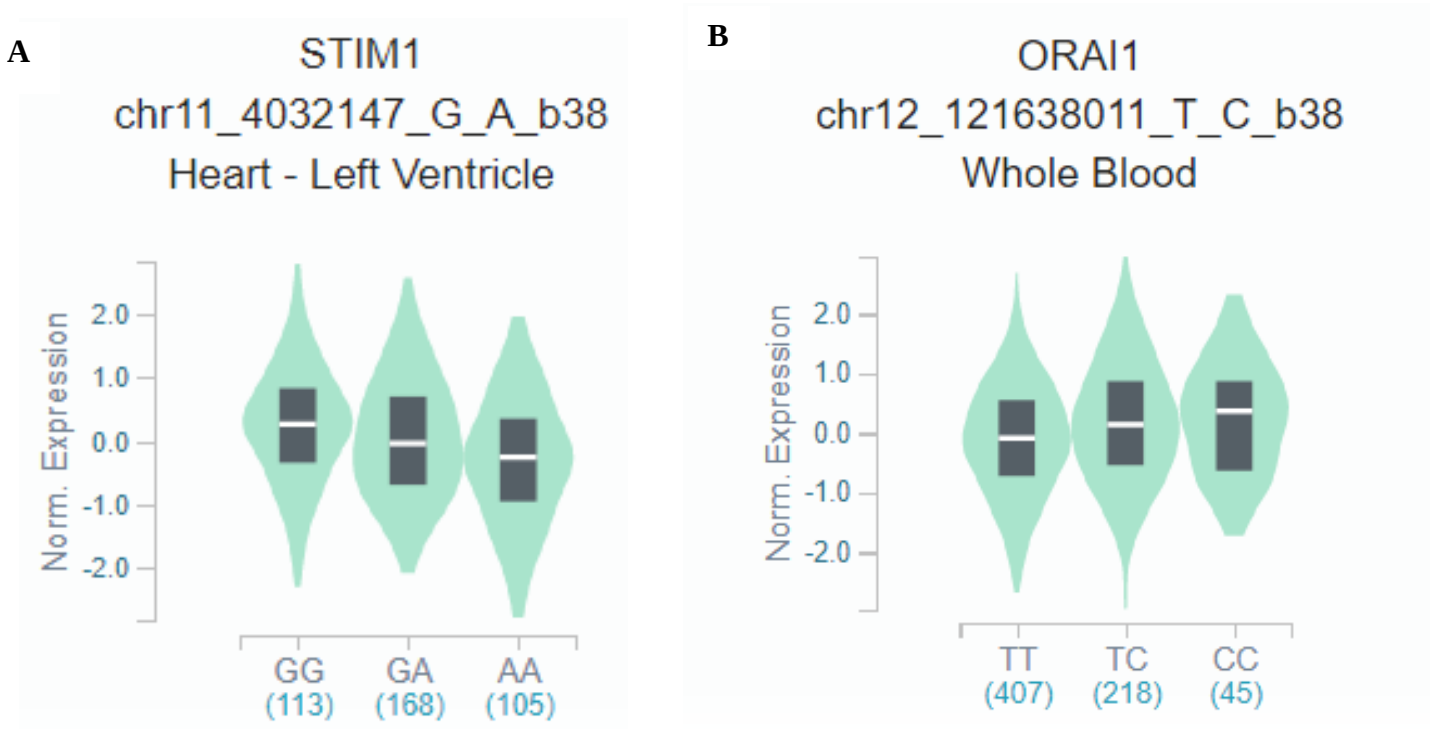


Figure S3. Correlation between genotype and expression in tissue determined by *Cis*-expression quantitative trait loci (*cis*-eQTLs). Figure **A** the expression of *STIM1* between rs7116520 Homozygous (GG), Heterozygous (AG), and Homozygous (AA) in heart-left ventricle tissue. Figure **B** the expression of *ORAI1* between rs6486795 Homozygous(CC), Heterozygous (TC) and Homozygous (TT) in whole blood.

Reviewer 2.

Comments and Suggestions for Authors

The manuscript 'Evaluation of genetic susceptibility between store-operated calcium influx pathway (STIM1 and ORAI1) and human hepatocellular carcinoma in patients with chronic hepatitis B infection' aims to identify polymorphisms associated with human hepatocellular carcinoma in 3631 Taiwanese patients. Overall, the manuscript presents data that are important to the field, yet a few points need to be clarified. The specific points that resulted in this conclusion are listed below.

Q1: Reviewer #2. The title is not reflective of the research presented in the manuscript. Genetic susceptibility is used in the title however the research is investigating association of SNP with human hepatocellular carcinoma not genetic susceptibility. Genetic susceptibility is also known as genetic predisposition, which cannot be properly evaluated in the current study. Title needs to be revised to reflect the presented study.

A1: Answer: We sincerely thank the reviewer's comments. Yes, genetic susceptibility is incorrect here. We have changed the title as "Evaluation for the Genetic Association Between Store-Operated Calcium Influx Pathway (STIM1 and ORAI1) and Human Hepatocellular Carcinoma in Patients with Chronic Hepatitis B Infection". In addition, to understand the role of store-operated calcium pathways (STIM1 and ORAI1) in HCC progression, functional studies by both total internal reflection fluorescence microscopy and transwell migration assay were applied. As shown in the Figure 2, we found that the colocolization of STIM1 and Orai1 was inhibited by 2-APB (SOC inhibitor). We further analyzed the effects of store-operated calcium pathways on cell migration in Huh 7 and HepG2 cell lines. The results showed that the cellular migration (Huh 7 and HepG2) significantly decreased by 2-APB (Figure 2B and 2C). Data has been added in revised manuscript [page 13, lines 307-325] [Pages 5-6, lines 141-171]. In addition, we added sentences in the part of discussion [Pages 15, lines 385-386] and conclusion [Pages 15-16, lines 394-399].

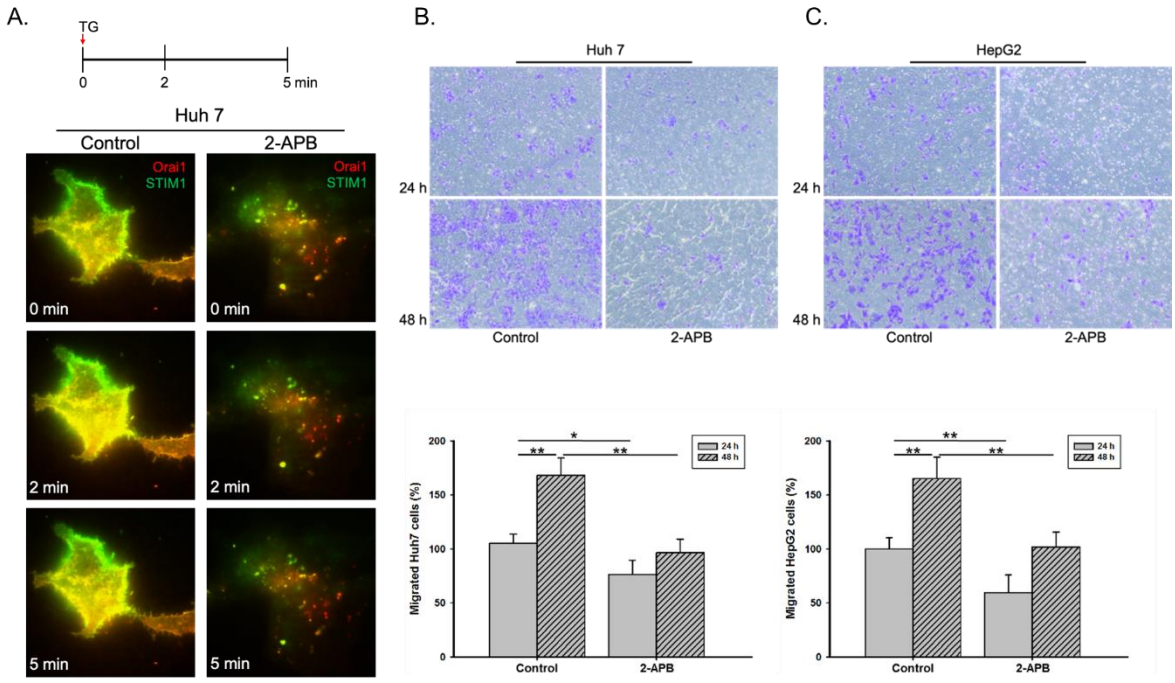


Figure 2. Inhibition of STIM1-Orai1 colocolization by 2-APB pre-treatment leads to reduced liver cancer cell migration ability. The Huh 7 cells were pre-treated with SOC inhibitor, 2-APB for 30 min. (A). The cells were co-transfected with STIM1-YFP and Orai1-mCherry and reseeded on Lab-Tek chambered cover glass for 24h. The inhibition effect of 2-APB on TG-induced SOC activity were observed with time-lapse TIRFM images. The inhibition effect of 2-APB on (B) Huh 7 or (C) HepG2 cell migration ability was examined by transwell migration assay. Statistically significant data are indicated by * for $p < 0.05$ and ** for $p < 0.01$.

Q2: Reviewer #2. Line 28: Remove 'of' from 'in total of 3631'.

A2:Thank you so much. We already Removed 'of' from 'in total of 3631' according to the reviewer's suggestions [Page 1, line 32].

Q3: Reviewer #2. Line 32-33. The authors state that 'our study revealed that calcium signaling is essential for hepatitis B virus replication'. This is not a valid conclusion of the study presented. While there is published research supporting this conclusion, the current study does not. The current study only evaluates SNP in *STIM1* and *ORAI1* and calcium signaling is not measured. Remove sentence from paragraph.

A3: Thank you so much for valuable comments. According to the suggestions, we provided cell-based studies as showed in the Figure 2. Inhibition of STIM1-Orai1 colocolization by 2-

APB pre-treatment leads to reduced liver cancer cell migration ability. Thus, images from total internal reflection fluorescence microscopy and data from transwell migration assay confirmed the critical roles of SOC-mediated signaling in the HCC migration.

Q4: Reviewer #2. Line 130: Specify which Qiagen kit was used.

A4: We thank the reviewer for the suggestions, the sentence has been added in the manuscript [Page 5, line 132].

Q5: Reviewer #2. Line 279-281: The authors state that 'We acknowledge a lack of calcium concentration data as a limitation of this study, which prevented us from understanding its correlation with HCC progression in CHB patients, even though a previous study revealed that the lowest Ca2+ level could be correlated with a decrease in HBV replication'. This is beyond the scope of the current study as the presented research does not evaluate calcium signaling. This sentence and similar statements in the discussion need to be removed.

A5: We are very grateful to the reviewer's suggestion. We already adjusted the sentences located in the last of discussion part as suggested by the reviewer. The modifications of sentences are presented in the following paragraph [Page 15, lines 385-391]. Here, our study also indicated that knockdown of SOC pathway prevented cell migration in two liver cancer (Huh 7 and HepG2) cell lines. We acknowledge that a weak correlation between *STIM1* and *ORAI1* polymorphisms and the risk of HCC progression in CHB patients may due to the modest sample size (3631 CHB patients), which led to a small power in the statistical analysis. Another possibility is that the majority of polymorphisms were located in the non-coding region. The genetic effects of each polymorphism to the expression of store-operated calcium channel are mild.

Q6: Reviewer #2. Font size varies throughout the entire manuscript. Keep font size consistent.

A6: We are very grateful to the reviewer's suggestions. We did it as suggested by the reviewer.

Q7: Reviewer #2. Table 2: Font of column titles is not consistent (minor allelic frequencies). Keep font consistent.

A7: Thank you for your suggestions. We did it as suggested by the reviewer.

Q8: Reviewer #2. Line 137: Change 'an' to 'a'.

A8: Many thanks to the reviewer's suggestions. We have made the correction according to the reviewer suggestions

Q9: Reviewer #2. Line 179: Define OR. OR is not defined until line 162.

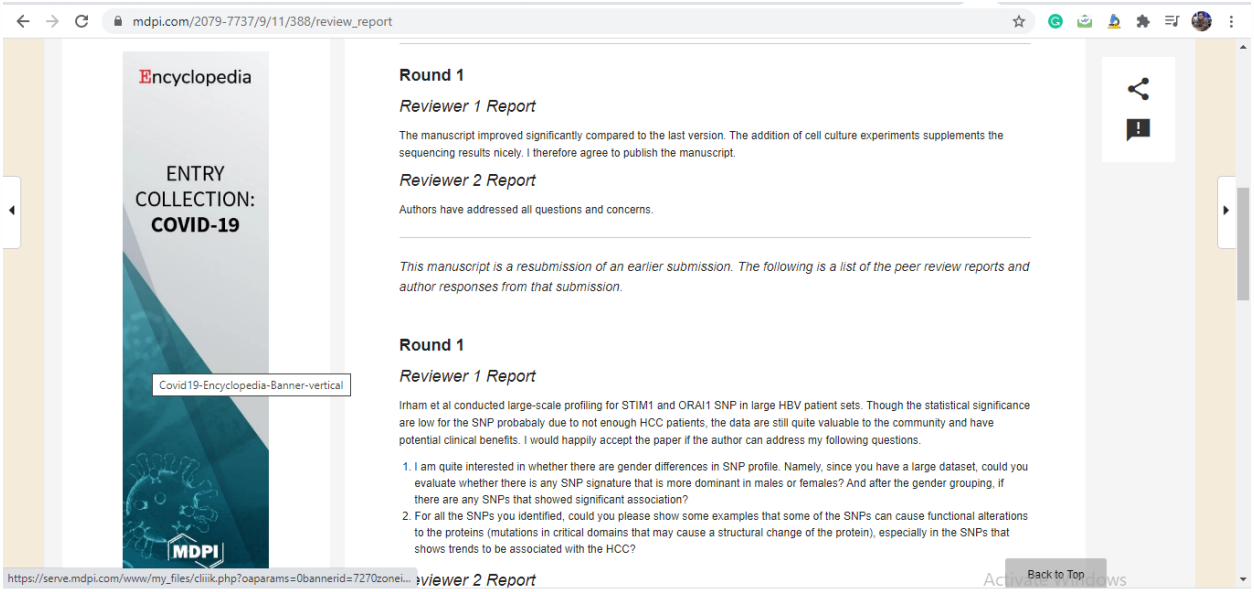
A9: Many thanks to the reviewer's suggestions. We have made the correction according to the comment from reviewer by adding the full name of OR, "odds ratio (OR)" [Page 6, line 179].

Q10: Reviewer #2. Line 233: Remove 'of' from 'in total of 3631'.

A10: We thank the reviewer for the suggestions. We have made the correction according to the reviewer's suggestion by removed 'of' from 'in total of 3631' [Page 14, line 340]. The revised sentences are as below: "In total 3631 patients with chronic hepatitis were recruited."

Recording process submission, reviewing and Publication

Online Review Report can be checked in the following link:
https://www.mdpi.com/2079-7737/9/11/388/review_report



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Reviewer 2 Report

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Author Response to Reviewer Comment

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Comments and Suggestions for Authors

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Answer: We sincerely thank the reviewer for the time taken to review our work.

Q1: Reviewer #1. I am quite interested in whether there are gender differences in SNP profile. Namely, since you have a large dataset, could you evaluate whether there is any SNP signature that is more dominant in males or females? And after the gender grouping, if there are any SNPs that showed significant association?

A1: We sincerely thank the reviewer for their suggestion. It is a very important point. As suggested by reviewer, we confirmed all the SNPs (40 SNPs) with stratified analyses in association between the SNPs and gender (male and female). In the present analyses showed that male gender is more dominant (2196 subjects) compared to female gender (1435 subjects) as showed in the following **Table**. In addition, we found that two SNPs (rs2959081 and rs7116520) of *STIM1* and one SNP of rs6486795 *ORAI1* gene showed an association for gender. The trend of these two *STIM1* SNPs (rs2959081 and rs7116520) showed an association in female gender, while the *ORAI1* SNP rs6486795 showed an association in male gender. Interestingly, through this analysis, we still identified the trend of two SNPs (rs7116520 and rs6486795) are consistent to be associated not only in HCC but also in case of gender. Furthermore, herein, we identified the new SNP of *ORAI1* rs2959081 showed the trend of significant association in male gender. However, after correction for multiple testing, none of the SNPs reached a significant level.

Associations of *STIM1* and *ORAI1* genes with gender (Male and Female)

SNP	Gene	Effect allele	Male Gender N=2196 (60.48%) (Genotype) *p* value[a] [q value]	Male Gender N=2196 (60.48%) (Dominant) *p* value[a] [q value]	Female Gender N=1435 (39.52%) (Genotype) *p* value[a] [q value]	Female Gender N=1435 (39.52%) (Dominant) *p* value[a] [q value]
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rs10835262	*STIM1*	A	0.913 [0.963]	0.908 [0.993]	0.920 [0.971]	0.828 [0.975]
rs4622250	*STIM1*	T	0.512 [0.963]	0.926 [0.993]	0.970 [0.971]	0.873 [0.975]
rs75197750	*STIM1*	T	0.196 [0.963]	0.131 [0.993]	0.668 [0.971]	0.380 [0.975]
rs10500589	*STIM1*	C	0.603 [0.963]	0.319 [0.993]	0.966 [0.971]	0.883 [0.975]
rs11030209	*STIM1*	C	0.642 [0.963]	0.926 [0.993]	0.905 [0.971]	0.761 [0.975]
rs10835270	*STIM1*	T	0.394 [0.963]	0.180 [0.993]	0.794 [0.971]	0.837 [0.975]
rs11030210	*STIM1*	T	0.729 [0.963]	0.945 [0.993]	0.381 [0.971]	0.479 [0.975]
rs7929653	*STIM1*	A	0.693 [0.963]	0.963 [0.993]	0.923 [0.971]	0.928 [0.975]
rs6578418	*STIM1*	G	0.240 [0.963]	0.095 [0.993]	0.328 [0.971]	0.249 [0.975]
rs10835272	*STIM1*	A	0.375 [0.963]	0.838 [0.993]	0.598 [0.971]	0.826 [0.975]

rs10742189	*STIM1*	C	0.808 [0.963]	0.755 [0.993]	0.404 [0.971]	0.232 [0.975]
rs7129444	*STIM1*	C	0.824 [0.963]	0.563 [0.993]	0.736 [0.971]	0.843 [0.975]
rs7118422	*STIM1*	T	0.926 [0.963]	0.929 [0.993]	0.715 [0.971]	0.419 [0.975]
rs4910863	*STIM1*	C	0.887 [0.963]	0.835 [0.993]	0.208 [0.971]	0.076 [0.975]
rs11030264	*STIM1*	G	0.757 [0.963]	0.476 [0.993]	0.183 [0.971]	0.068 [0.975]
rs7924984	*STIM1*	A	0.389 [0.963]	0.506 [0.993]	0.240 [0.971]	0.319 [0.975]
rs2412338	*STIM1*	T	0.396 [0.963]	0.501 [0.993]	0.163 [0.971]	0.358 [0.975]
rs10835402	*STIM1*	C	0.963 [0.963]	0.790 [0.993]	0.109 [0.971]	0.170 [0.975]
rs11030472	*STIM1*	G	0.147 [0.963]	0.078 **[**0.993**]**	0.456 [0.971]	0.214 [0.975]
rs11030478	*STIM1*	A	0.688 [0.963]	0.934 [0.993]	0.920 [0.971]	0.824 [0.975]
rs11030486	*STIM1*	T	0.698 [0.963]	0.874 [0.993]	0.926 [0.971]	0.823 [0.975]
rs12284835	*STIM1*	A	0.071 [0.963]	0.109 [0.993]	0.822 [0.971]	0.636 [0.975]
rs727152	*STIM1*	G	0.519 [0.963]	0.315 [0.993]	0.725 [0.971]	0.951 [0.975]
rs2959081	*STIM1*	C	0.608 [0.963]	0.543 [0.993]	**0.021** [0.971]	**0.005** [0.975]
rs11030639	*STIM1*	G	0.770 [0.963]	0.666 [0.993]	0.054 [0.971]	0.195 [0.975]
rs7116520	*STIM1*	G	0.410 [0.963]	0.209 [0.993]	**0.007** [0.971]	**0.001** [0.975]
rs1442725	*STIM1*	T	0.304 [0.963]	0.373 [0.993]	0.706 [0.971]	0.467 [0.975]
rs11030841	*STIM1*	A	0.611 [0.963]	0.486 [0.993]	0.381 [0.971]	0.885 [0.975]
rs4910882	*STIM1*	A	0.637 [0.963]	0.501 [0.993]	0.362 [0.971]	0.975 [0.975]
rs6486795	*ORAI1*	C	0.078 [0.963]	**0.037** **[**0.993**]**	0.971 [0.971]	0.850 [0.975]
rs74936888	*ORAI1*	C	0.304 [0.963]	0.124 **[**0.993**]**	0.419 [0.971]	0.418 [0.975]
rs3741595	*ORAI1*	C	0.184 [0.963]	0.081 [0.993]	0.731 [0.971]	0.637 [0.975]

[a] Adjusted by alanine transaminase and age. Significant *p* values are in **bold***. The q value is the false discovery rate (FDR) estimation for multi-testing.

Q2: Reviewer #1. For all the SNPs you identified, could you please show some examples that some of the SNPs can cause functional alterations to the proteins (mutations in critical domains that may cause a structural change of the protein), especially in the SNPs that shows trends to be associated with the HCC?

A2: We sincerely thank the reviewer for the time taken to review our work and the important suggestions are given**.** According to reviewer suggestion**,** we try to confirm the effect of the alteration/ deleteriousness of variants in protein by using some of the functional annotation tools such as the scale-invariant feature transform (SIFT), **Poly**morphism **Phen**otyping v2 **(**PlyPhen-2), ClinVar and Combined Annotation Dependent Depletion (CADD) databases. As shown in the following **Table**, all four SNPs (*STIM1* rs6578418, rs11030472, rs7116520, and *ORAI1* rs6486795) are intron variants and we could not find the information of these four SNPs (the SNPs that shows trends to be associated with the HCC) effect of the change on protein function in three databases (SIFT, PlyPhen-2, and ClinVar). However, we still identified the information of four SNPs from CADD. The scores of CADD are displayed in four variants with score less than 30 which mean those four variants more likely to be benign rather than deleterious.

Information about the variants consequence from various databases

SNP	Position (hg38) (bp)	Effect Allele	Consequence Type	PolyPhen2	SIFT	ClinVar	CADD
rs6578418	Chr11:3905002	G	Intron variant	NA	NA	NA	4.363(Benign)
rs11030472	Chr11:3978105	G	Intron variant	NA	NA	NA	8.781(Benign)
rs7116520	Chr11:4032147	G	Intron variant	NA	NA	NA	0.541(Benign)
rs6486795	Chr12:121638011	C	Intron variant	NA	NA	NA	0.481(Benign)

NA: Not Available

Additionally, we try to elaborate the relationship between the *STIM1* rs6578418, rs11030472, rs7116520 and *ORAI1* rs6486795 and gene expression. Through this way, we utilized the publicly available databases GTEx portal (http:// www.gtexportal.org/home/) to confirmed the tissue expression quantitative trait loci. For two *STIM1* SNPs rs6578418 and rs11030472, we could not find the expression data on the GTEx portal database due to limited available tissue (**Table S3**). While the trend of *STIM1* rs7116520 showed in a variety of tissues including heart-left ventricle and nerve tibial lung tissue with GG genotype had higher expression compared to those with AG and AA genotype (**Figure S3**). Furthermore, *ORAI1* rs6486795 with CC genotype showing a highly expressed compared to those with TC and TT genotype in variety of tissues (*e.g.* whole blood, esophagus, thyroid, heart, stomach, pancreas, muscle, colon). This paragraph has been added in the result part of this study [Page 13, lines 298-308] and we also added the SNP annotation data query in the method section [Page 5, lines 138-141]. Furthermore, the Table (**Table S3**) and Figure (**Figure S3**) were added in the supplementary materials [Page 15, lines, 409-410 and page 16, lines 417-418].

Table S3. Expression Quantitative trail loci (eQTL) results of The SNP from Genotype-tissue expression (GTEx).

SNP ID	Gencode ID (ENSG00000-)	Gene symbol	p value	Effect size	Tissue	Actions
rs7116520	167323.11	*STIM1*	1.1e-9	-0.18	Nerve - Tibial	GG>AG>AA
	167323.11	*STIM1*	2.0e-8	-0.22	Heart-Left Ventricle	GG>AG>AA
rs6578418	NA	NA	NA	NA	NA	NA
rs11030472	NA	NA	NA	NA	NA	NA
rs6486795	276045.2	*ORAI1*	1.30E-17	0.22	Whole Blood	CC>CT>TT
	276045.2	*ORAI1*	9.90E-16	0.29	Esophagus - Mucosa	CC>CT>TT
	276045.2	*ORAI1*	3.30E-13	0.23	Thyroid	CC>CT>TT
	276045.2	*ORAI1*	7.60E-08	0.24	Stomach	CC>CT>TT
	276045.2	*ORAI1*	4.50E-07	0.25	Pancreas	CC>CT>TT
	276045.2	*ORAI1*	4.80E-07	0.11	Muscle - Skeletal	CC>CT>TT
	276045.2	*ORAI1*	1.5E-06	0.21	Colon - Transverse	CC>CT>TT
	276045.2	*ORAI1*	0.000029	0.19	Esophagus - Gastroesophageal Junction	CC>CT>TT
	276045.2	*ORAI1*	0.000058	0.17	Heart - Atrial Appendage	CC>CT>TT
	276045.2	*ORAI1*	0.00016	0.13	Esophagus - Muscularis	CC>CT>TT

Source: Expression Quantitative trail loci (eQTL) obtained from https://gtexportal.org/home, NA: Not Available

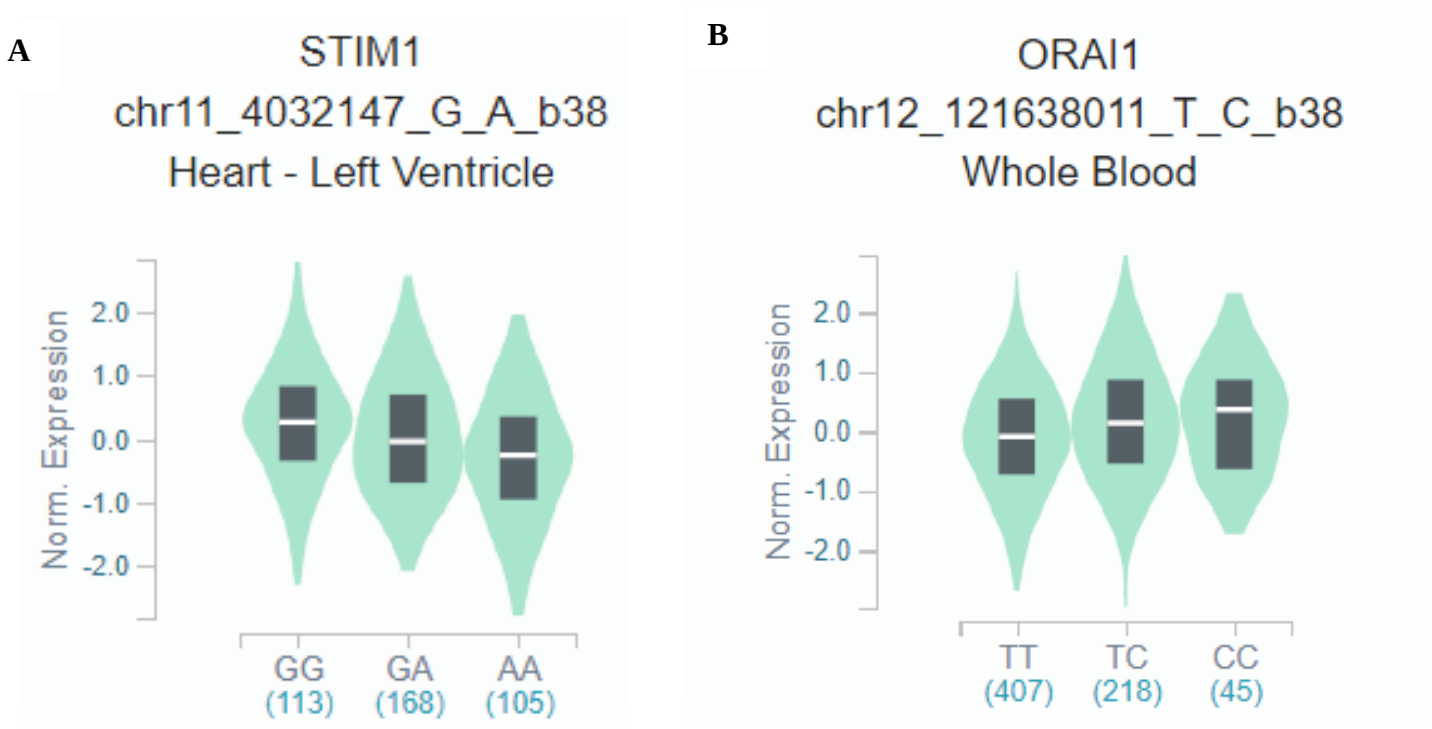

Figure S3. Correlation between genotype and expression in tissue determined by *Cis*-expression quantitative trait loci (*cis*-eQTLs). Figure **A** *STIM1* rs7116520 Homozygous (GG) >Heterozygous (AG)>Homozygous (AA) in heart-left ventricle tissue. Figure **B** *ORAI1* rs6486795 Homozygous CC>Heterozygous (TC)>Homozygous (TT) in whole blood.

Reviewer 2.

Comments and Suggestions for Authors

The manuscript 'Evaluation of genetic susceptibility between store-operated calcium influx pathway (STIM1 and ORAI1) and human hepatocellular carcinoma in patients with chronic hepatitis B infection' aims to identify polymorphisms associated with human hepatocellular carcinoma in 3631 Taiwanese patients. Overall, the manuscript presents data that are important to the field, yet a few points need to be clarified. The specific points that resulted in this conclusion are listed below.

Answer: We sincerely thank the reviewer for the time taken to review our work

Q1: Reviewer #2. The title is not reflective of the research presented in the manuscript. Genetic susceptibility is used in the title however the research is investigating association of SNP with human hepatocellular carcinoma not genetic susceptibility. Genetic susceptibility is also known as genetic predisposition, which cannot be properly evaluated in the current study. Title needs to be revised to reflect the presented study.

A1: We thank the reviewer for the suggestions. To reflect the title of this study, we currently evaluated the Store-Operated Calcium (STIM1 and ORAI1) as the additional supported data of functional cell-based assay. As shown in the current study (**Figure 2**) that we validated the roles of Store-Operated Calcium influx pathway (STIM1 and ORAI1) in HCC cell progression using two liver cancer cell lines (Huh 7 and HepG2). **Figure 2A** showed that the colocolization of STIM1 and Orai1 was inhibited by 2-APB pre-treatment 30 min in Huh 7 cells. We further analyzed the inhibition effect of Store-Operated Calcium activity suppression on cell migration in Huh 7 and HepG2 cell lines. The results showed that liver cancer cells migration (Huh 7 and HepG2) significantly decreased by 2-APB pre-treatment compared to the control group (**Figure 2B and 2C**). These results indicated that inhibition of Store-Operated Calcium by 2-APB was able to block the liver cancer cells migration. This paragraph has been added in the result part of this study [page 13, lines 310-328] and we also added the functional cell-based assay accordingly in the method section [Pages 5-6, lines 143-173]. In addition, we added the sentences in the discussion part [Pages 15, lines 388-390] and modified the conclusion [Pages 15-16, lines 397-402].

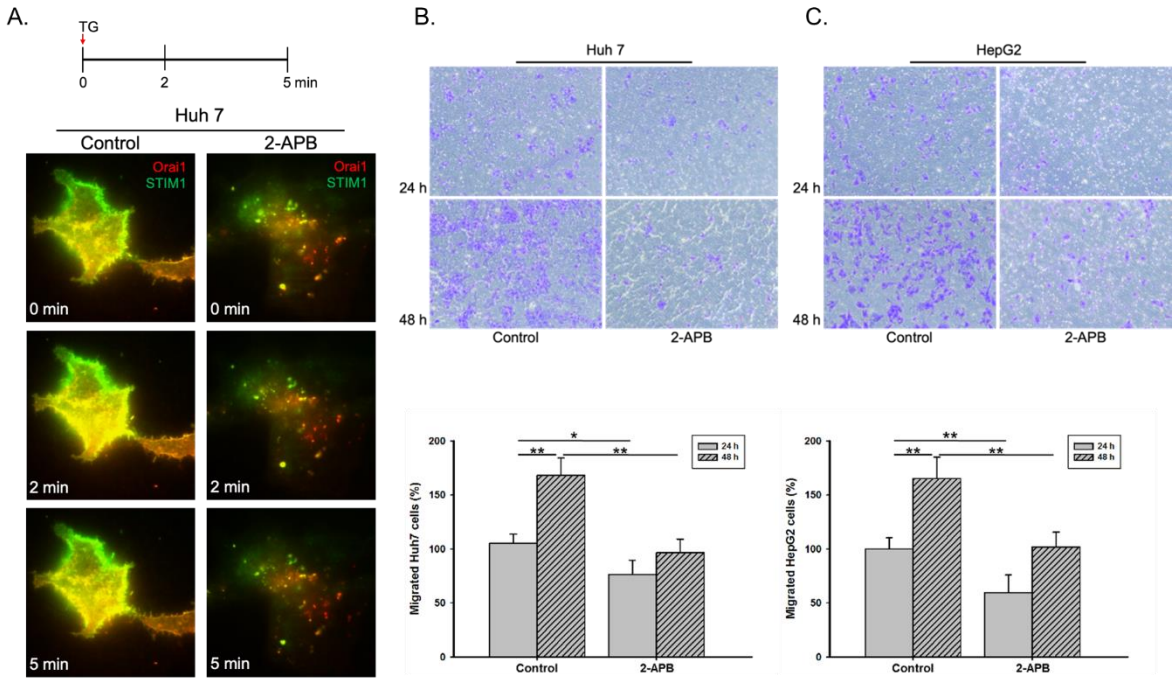

Figure 2. Inhibition of STIM1-Orai1 colocolization by 2-APB pre-treatment leads to reduced liver cancer cell migration ability. The Huh 7 cells were pre-treated with SOC inhibitor, 2-APB for 30 min. (A). The cells were co-transfected with STIM1-YFP and Orai1-mCherry and reseeded on Lab-Tek chambered cover glass for 24h. The inhibition effect of 2-APB on TG-induced SOC activity were observed with time-lapse TIRFM images. The inhibition effect of 2-APB on (B) Huh 7 or (C) HepG2 cell migration ability was examined by transwell migration assay. Statistically significant data are indicated by * for $p < 0.05$ and ** for $p < 0.01$.

Q2: Reviewer #2. Line 28: Remove 'of' from 'in total of 3631'.
A2: We sincerely thank the reviewer for their suggestion. It is a very important point. We already Removed 'of' from 'in total of 3631' according to the reviewer's suggestions [Page 1, line 31].

Q3: Reviewer #2. Line 32-33. The authors state that 'our study revealed that calcium signaling is essential for hepatitis B virus replication'. This is not a valid conclusion of the study presented. While there is published research supporting this conclusion, the current study does not. The current study only evaluates SNP in *STIM1* and *ORAI1* and calcium signaling is not measured. Remove sentence from paragraph.

A3: We sincerely thank the reviewer for taking the time to review our work. According to the reviewer comment, we provided the data of functional cell-based study as showed in the **Figure 2**. Functional studies by both total internal reflection fluorescence microscopy and transwell migration assay confirmed the critical roles of SOC-mediated signaling in the HCC migration. According to the result of current data of functional cell-based assay, we corrected the sentences as presented in the following paragraph [Page 1, lines 35-41].

In particular, our study revealed that calcium (Ca^{2+}) signaling is essential for the migration of HCC, however, genetic polymorphisms of SOC pathway (STIM1 and ORAI1) are not significantly associated with HCC progression. Besides, three SNPs of *STIM1* (rs6578418, rs11030472, and rs7116520) and one SNP of *ORAI1* (rs6486795) with *a borderline significant* trend, might be worth further study. Based on such a comprehensively screening in 3631 patients with chronic hepatitis, we believe that our results are of substantial interest to geneticists especially population geneticists, and clinical physicians.

Furthermore, we modified the conclusion of this study as presented in the following paragraph [Pages 15-16, lines 397-402].

Our study indicated that inhibition of SOC is able to block the cell migration in two liver cancer (Huh 7 and HepG2) cell lines. Although Ca^{2+} signaling is essential for the development of HCC, the genetic polymorphisms of SOC pathway (*STIM1* and *ORAI1*) are not significantly associated with HCC progression after multiple correction. Besides, three SNPs of *STIM1* (rs6578418, rs11030472, and rs7116520) and one SNP of *ORAI1* (rs6486795) with *a borderline significant* trend should be particularly focused in the future study.

Q4: Reviewer #2. Line 130: Specify which Qiagen kit was used.

A4: We thank the reviewer for the suggestions, the sentence has been added in the manuscript [Page 5, line 134].

Q5: Reviewer #2. Line 279-281: The authors state that 'We acknowledge a lack of calcium concentration data as a limitation of this study, which prevented us from understanding its correlation with HCC progression in CHB patients, even though a previous study revealed that the lowest Ca2+ level could be correlated with a decrease in HBV replication'. This is beyond the scope of the current study as the presented research does not evaluate calcium signaling. This sentence and similar statements in the discussion need to be removed.

A5: We are very grateful to the reviewer's suggestion. We already adjusted the sentences located in the last of discussion part as suggested by the reviewer. The modifications of sentences are presented in the following paragraph [Page 15, lines 390-394].

We acknowledge a weak correlation between *STIM1* and *ORAI1* polymorphisms and the risk of HCC progression in CHB patients may have been due to the modest sample size (3631 CHB patients), which led to a small power in the statistical analysis. Another possibility is that the majority of polymorphisms were located in the non-coding region. The genetic effects of each polymorphism to the expression of store-operated calcium channel are mild.

Q6: Reviewer #2. Font size varies throughout the entire manuscript. Keep font size consistent.
A6: We are very grateful to the reviewer's suggestion. We did it as suggested by the reviewer.

Q7: Reviewer #2. Table 2: Font of column titles is not consistent (minor allelic frequencies). Keep font consistent.
A7: Thank you for your suggestion. We did it as suggested by the reviewer.

Q8: Reviewer #2. Line 137: Change 'an' to 'a'.
A8: Many thanks to the reviewer's suggestions. We have made the correction according to the reviewer suggestion by change "an" to "a" in the sentences [Page 6, line 180]. The revised sentences are as below:

Linkage disequilibrium (LD) was evaluated with Haploview software version. 4.2 (Broad Institute, Cambridge, MA, USA) by a SNP analysis of *STIM1* and *ORAI1* polymorphisms together as haplotype blocks.

Q9: Reviewer #2. Line 139: Define OR. OR is not defined until line 162.
A9: Many thanks to the reviewer's suggestions. We have made the correction according to the comment from reviewer by adding the full name of OR, "odds ratio (OR)" [Page 6, line 182].

Q10: Reviewer #2. Line 233: Remove 'of' from 'in total of 3631'.
A10: We thank the reviewer for the suggestions. We have made the correction according to the reviewer's suggestion by removed 'of' from 'in total of 3631' [Page 14, line 343]. The revised sentences are as below:
"In total 3631 patients with chronic hepatitis were recruited."

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Dear Mr. Muhammad Irham,

Thank you very much for submitting your manuscript to Biology:

Journal name: Biology
Manuscript ID: biology-947101
Type of manuscript: Article
Title: Evaluation of Genetic susceptibility between store-operated calcium influx pathway (STIM1 and ORAI1) and human Hepatocellular Carcinoma in Patients with Chronic Hepatitis B Infection
Authors: Lalu Muhammad Irham, Wan Hsuan Chou, Wirawan Adikusuma, Henry Sung-Ching Wong, Dyah Aryani Perwitasari, Ben Kuen Chen *, Hwai I Yang *, Wei Chiao Chang *
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Dear Mr. Muhammad Irham,

Your manuscript has been assigned to Amy Tian for further processing who will act as a point of contact for any questions related to your paper.

Journal: Biology
Manuscript ID: biology-947101
Title: Evaluation of Genetic susceptibility between store-operated calcium influx pathway (STIM1 and ORAI1) and human Hepatocellular Carcinoma in Patients with Chronic Hepatitis B Infection
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[Biology] Manuscript ID: biology-947101 - Major Revisions (by 20 October)

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Amy Tian <amy.tian@mdpi.com> Mon, Oct 12, 2020 at 10:30 PM
Reply-To: amy.tian@mdpi.com
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Reply-To: amy.tian@mdpi.com
To: Wei-Chiao Chang <weichiao.chang@gmail.com>
Cc: Lalu Muhammad Irham <lalu.irham@pharm.uad.ac.id>, Wan Hsuan Chou <ocean.chou@tmu.edu.tw>, Wirawan Adikusuma <adikusuma28@gmail.com>, Henry Sung-Ching Wong <miningyue@gmail.com>, Dyah Aryani Perwitasari <diahperwitasari2003@yahoo.com>, Ben Kuen Chen <bkchen58@mail.ncku.edu.tw>, Hwai I Yang <hiyang@gate.sinica.edu.tw>, Biology Editorial Office <biology@mdpi.com>

Dear Dr. Chang,

I hope this email finds you well. This is the follow up with my message on 21 October 2020.

We are writing as we would like to know your revision progress and the possible time that it could be submitted? As the manuscript will be pending major revision for more time, we would like to ask you to upload the revised version to the online system as a new submission and it will be assigned a new manuscript ID.

http://www.mdpi.com/user/manuscripts/upload/?journal=biology

We will retain all records of biology-947101. The new submission will be sent to the previous reviewers directly once we receive it.

Thank you very much for your consideration.

Looking forward to hearing from you soon.

Kind regards,
Ms. Amy Tian
Assistant Editor

Biology Impact Factor in 2020: 3.796 (2019 JCR®). For joining the Editorial Board, please contact with biology@mdpi.com
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On 2020/10/21 9:42, Amy Tian wrote:

> Dear Dr. Chang,
>
> Thank you very much for your kind reply.
>
> We can give enough time for you to do your studies.
>
> Meanwhile, I have a proposal. As the manuscript will be pending major revision for more time, we would like to ask you to upload the revised version to the online system as a new submission and it will be assigned a new manuscript ID.
>
> http://www.mdpi.com/user/manuscripts/upload/?journal=biology

We will retain all records of biology-947101. The new submission will
be sent to the previous reviewers directly once we receive it.

Thank you very much for your consideration.

Looking forward to hearing from you soon.

Kind regards,
Ms. Amy Tian
Assistant Editor

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Editorial Board, please contact with biology@mdpi.com

On 2020/10/20 19:37, Wei-Chiao Chang wrote:

> Dear Amy,
>
> We are doing cell-based functional studies to improve the quality of this manuscript.
> Could you please extend the deadline of revision ?
> Hopefully, we will send it back next week.
> ____Thank you~
>
> wei chiao
>
> ---
>
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>
> 臺北學大學藥學院
>
> Wei-Chiao Chang (D.Phil.; Oxon)
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> Distinguished Professor/Vice Dean, School of Pharmacy
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> Taipei Medical University, Taiwan
>
> Tel: 886-2-27361661 ext.6187 <tel:02%202736%201661>
>
> http://twsnp.tmu.edu.tw/
>
> Amy Tian <amy.tian@mdpi.com <mailto:amy.tian@mdpi.com>> 於 2020年10月 19 日 週一 下午4:29寫道:
>
> > Dear Mr. Muhammad Irham,
> >
> > We sent a revision request for the following manuscript on 13
> > October 2020.
> >
> > Manuscript ID: biology-947101
> > Type of manuscript: Article
> > Title: Evaluation of Genetic susceptibility between store-operated

calcium
influx pathway (STIM1 and ORAI1) and human Hepatocellular Carcinoma in
Patients with Chronic Hepatitis B Infection
Authors: Lalu Muhammad Irham, Wan Hsuan Chou, Wirawan Adikusuma, Henry
Sung-Ching Wong, Dyah Aryani Perwitasari, Ben Kuen Chen *, Hwai I
Yang *, Wei
Chiao Chang *
Received: 12 September 2020
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Thank you in advance for your kind cooperation and we look forward
to hearing
from you soon.

Kind regards,
Ms. Amy Tian
Assistant Editor
--
MDPI Beijing Office Tongzhou, Jincheng Center, Room 2207, Tongzhou
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China

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Wei-Chiao Chang <weichiao.chang@gmail.com> Fri, Oct 23, 2020 at 1:15 AM
To: amy.tian@mdpi.com
Cc: Lalu Muhammad Irham <lalu.irham@pharm.uad.ac.id>, Wan Hsuan Chou <ocean.chou@tmu.edu.tw>, Wirawan Adikusuma <adikusuma28@gmail.com>, Henry Sung-Ching Wong <miningyue@gmail.com>, Dyah Aryani Perwitasari <diahperwitasari2003@yahoo.com>, Ben Kuen Chen <bkchen58@mail.ncku.edu.tw>, Hwai I Yang <hiyang@gate.sinica.edu.tw>, Biology Editorial Office <biology@mdpi.com>

Hi Amy,
Many thanks for your mail.
I will re-submit our manuscript on 28th Oct.

Best regards,

wei chiao

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LALU MUHAMMAD IRHAM <lalu.irham@pharm.uad.ac.id>

[Biology] Manuscript ID: biology-998991 - Accepted for Publication

1 message

Amy Tian <amy.tian@mdpi.com> Thu, Nov 5, 2020 at 7:50 PM
Reply-To: Amy Tian <amy.tian@mdpi.com>, Biology Editorial Office <biology@mdpi.com>
To: Lalu Muhammad Irham <lalu.irham@pharm.uad.ac.id>
Cc: Wan-Hsuan Chou <s700081@gmail.com>, Yu-Shiuan Wang <yswang1004@gmail.com>, Wirawan Adikusuma <adikusuma28@gmail.com>, Henry Sung-Ching Wong <miningyue@gmail.com>, Dyah Aryani Perwitasari <diahperwitasari2003@yahoo.com>, Wan Chen Huang <wanchen.huang@gmail.com>, Ben Kuen Chen <bkchen58@icloud.com>, Hwai I Yang <hiyang@gate.sinica.edu.tw>, Wei Chiao Chang <weichiao.chang@gmail.com>, Biology Editorial Office <biology@mdpi.com>, Amy Tian <amy.tian@mdpi.com>

Dear Mr. Muhammad Irham,

We are pleased to inform you that the following paper has been officially accepted for publication:

Manuscript ID: biology-998991
Type of manuscript: Article
Title: Evaluation for the Genetic Association Between Store-Operated Calcium Influx Pathway (STIM1 and ORAI1) and Human Hepatocellular Carcinoma in Patients with Chronic Hepatitis B Infection
Authors: Lalu Muhammad Irham, Wan-Hsuan Chou, Yu-Shiuan Wang, Wirawan Adikusuma, Henry Sung-Ching Wong, Dyah Aryani Perwitasari, Wan Chen Huang, Ben Kuen Chen *, Hwai I Yang *, Wei Chiao Chang *
Received: 28 October 2020
E-mails: lalu.irham@pharm.uad.ac.id, s700081@gmail.com, yswang1004@gmail.com, adikusuma28@gmail.com, miningyue@gmail.com, diahperwitasari2003@yahoo.com, wanchen.huang@gmail.com, bkchen58@icloud.com, hiyang@gate.sinica.edu.tw, weichiao.chang@gmail.com
Submitted to section: Genetics and Genomics,
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We will now make the final preparations for publication, then return the manuscript to you for your approval.

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LALU MUHAMMAD IRHAM <lalu.irham@pharm.uad.ac.id>

[Biology] Manuscript ID: biology-998991 - Final Proofreading Before Publication

4 messages

Amy Tian <amy.tian@mdpi.com> Fri, Nov 6, 2020 at 2:22 AM
Reply-To: amy.tian@mdpi.com
To: Lalu Muhammad Irham <lalu.irham@pharm.uad.ac.id>
Cc: Wan-Hsuan Chou <s700081@gmail.com>, Yu-Shiuan Wang <yswang1004@gmail.com>, Wirawan Adikusuma <adikusuma28@gmail.com>, Henry Sung-Ching Wong <miningyue@gmail.com>, Dyah Aryani Perwitasari <diahperwitasari2003@yahoo.com>, Wan Chen Huang <wanchen.huang@gmail.com>, Ben Kuen Chen <bkchen58@icloud.com>, Hwai I Yang <hiyang@gate.sinica.edu.tw>, Wei Chiao Chang <weichiao.chang@gmail.com>, Biology Editorial Office <biology@mdpi.com>

Dear Mr. Muhammad Irham,

We invite you to proofread your manuscript to ensure that this is the final version that can be published and confirm that you will require no further changes from hereon:

Manuscript ID: biology-998991
Type of manuscript: Article
Title: Evaluation for the Genetic Association Between Store-Operated Calcium Influx Pathway (STIM1 and ORAI1) and Human Hepatocellular Carcinoma in Patients with Chronic Hepatitis B Infection
Authors: Lalu Muhammad Irham, Wan-Hsuan Chou, Yu-Shiuan Wang, Wirawan Adikusuma, Henry Sung-Ching Wong, Dyah Aryani Perwitasari, Wan Chen Huang, Ben Kuen Chen *, Hwai I Yang *, Wei Chiao Chang *
Received: 28 October 2020
E-mails: lalu.irham@pharm.uad.ac.id, s700081@gmail.com, yswang1004@gmail.com, adikusuma28@gmail.com, miningyue@gmail.com, diahperwitasari2003@yahoo.com, wanchen.huang@gmail.com, bkchen58@icloud.com, hiyang@gate.sinica.edu.tw, weichiao.chang@gmail.com
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[Biology] Manuscript ID: biology-998991 - Manuscript Resubmitted

1 message

Submission System <submission@mdpi.com> Sun, Nov 8, 2020 at 9:19 PM
Reply-To: Amy Tian <amy.tian@mdpi.com>, Biology Editorial Office <biology@mdpi.com>
To: Lalu Muhammad Irham <lalu.irham@pharm.uad.ac.id>
Cc: Wan-Hsuan Chou <s700081@gmail.com>, Yu-Shiuan Wang <yswang1004@gmail.com>, Wirawan Adikusuma <adikusuma28@gmail.com>, Henry Sung-Ching Wong <miningyue@gmail.com>, Dyah Aryani Perwitasari <diahperwitasari2003@yahoo.com>, Wan Chen Huang <wanchen.huang@gmail.com>, Ben Kuen Chen <bkchen58@icloud.com>, Hwai I Yang <hiyang@gate.sinica.edu.tw>, Wei Chiao Chang <weichiao.chang@gmail.com>

Dear Mr. Muhammad Irham,

Thank you very much for resubmitting the modified version of the following manuscript:

Manuscript ID: biology-998991
Type of manuscript: Article
Title: Evaluation for the Genetic Association Between Store-Operated Calcium Influx Pathway (STIM1 and ORAI1) and Human Hepatocellular Carcinoma in Patients with Chronic Hepatitis B Infection
Authors: Lalu Muhammad Irham, Wan-Hsuan Chou, Yu-Shiuan Wang, Wirawan Adikusuma, Henry Sung-Ching Wong, Dyah Aryani Perwitasari, Wan Chen Huang, Ben Kuen Chen *, Hwai I Yang *, Wei Chiao Chang *
Received: 28 October 2020
E-mails: lalu.irham@pharm.uad.ac.id, s700081@gmail.com, yswang1004@gmail.com, adikusuma28@gmail.com, miningyue@gmail.com, diahperwitasari2003@yahoo.com, wanchen.huang@gmail.com, bkchen58@icloud.com, hiyang@gate.sinica.edu.tw, weichiao.chang@gmail.com
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LALU MUHAMMAD IRHAM <lalu.irham@pharm.uad.ac.id>

[Biology] Manuscript ID: biology-998991; doi: 10.3390/biology9110388. Paper has been published.

2 messages

Biology Editorial Office <biology@mdpi.com> Mon, Nov 9, 2020 at 4:04 AM
Reply-To: Amy Tian <amy.tian@mdpi.com>, Biology Editorial Office <biology@mdpi.com>
To: Lalu Muhammad Irham <lalu.irham@pharm.uad.ac.id>
Cc: Biology Editorial Office <biology@mdpi.com>, Amy Tian <amy.tian@mdpi.com>

Dear Mr. Irham,

We are pleased to inform you that "Evaluation for the Genetic Association between Store-Operated Calcium Influx Pathway (STIM1 and ORAI1) and Human Hepatocellular Carcinoma in Patients with Chronic Hepatitis B Infection" by Lalu Muhammad Irham, Wan-Hsuan Chou, Yu-Shiuan Wang, Wirawan Adikusuma, Henry Sung-Ching Wong, Dyah Aryani Perwitasari, Wan-Chen Huang, Ben-Kuen Chen *, Hwai-I Yang *, Wei-Chiao Chang * has been published in Biology and is available online:

Abstract: https://www.mdpi.com/2079-7737/9/11/388
HTML Version: https://www.mdpi.com/2079-7737/9/11/388/htm
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Cc: billing@mdpi.com, website@mdpi.com, biology@mdpi.com, amy.tian@mdpi.com

Dear Authors,

We are pleased to inform you that your article "Evaluation for the Genetic Association between Store-Operated Calcium Influx Pathway (STIM1 and ORAI1) and Human Hepatocellular Carcinoma in Patients with Chronic Hepatitis B Infection" has been published in Biology and is available online:

Abstract: https://www.mdpi.com/2079-7737/9/11/388
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Payment Receipt:

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Name of co-authors:	Lalu Muhammad Irham, Wan-Hsuan Chou, Yu-Shiuan Wang, Wirawan Adikusuma, Henry Sung-Ching Wong, Dyah Aryani Perwitasari, Wan Chen Huang, Ben Kuen Chen, Hwai I Yang and Wei Chiao Chang Additional Author Information
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Evaluation for the Genetic Association Between Store-Operated Calcium Influx Pathway (STIM1 and ORAI1) and Human Hepatocellular Carcinoma in Patients with Chronic Hepatitis B Infection

Authored by:

Lalu Muhammad Irham; Wan-Hsuan Chou; Yu-Shiuan Wang; Wirawan Adikusuma; Henry Sung-Ching Wong; Dyah Aryani Perwitasari; Wan Chen Huang; Ben Kuen Chen; Hwai I Yang; Wei Chiao Chang

has been accepted in *Biology* (ISSN 2079-7737) on 06 November 2020

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Article

Evaluation for the Genetic Association between Store-Operated Calcium Influx Pathway (STIM1 and ORAI1) and Human Hepatocellular Carcinoma in Patients with Chronic Hepatitis B Infection

Lalu Muhammad Irham [1,2], Wan-Hsuan Chou [1,3], Yu-Shiuan Wang [4,5], Wirawan Adikusuma [1,6], Henry Sung-Ching Wong [1,3], Dyah Aryani Perwitasari [2], Wan-Chen Huang [4], Ben-Kuen Chen [7,8,*], Hwai-I Yang [9,10,*] and Wei-Chiao Chang [1,3,11,12,*]

1 Department of Clinical Pharmacy, School of Pharmacy, College of Pharmacy, Taipei Medical University, Taipei 11031, Taiwan; lalu.irham@pharm.uad.ac.id (L.M.I.); s700081@gmail.com (W.-H.C.); adikusuma28@gmail.com (W.A.); miningyue@gmail.com (H.S.-C.W.)
2 Faculty of Pharmacy, University of Ahmad Dahlan, Yogyakarta 55164, Indonesia; diahperwitasari2003@yahoo.com
3 Master Program for Clinical Pharmacogenomics and Pharmacoproteomics, School of Pharmacy, Taipei Medical University, Taipei 11031, Taiwan
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5 Ph.D Program in Clinical Drug Development of Chinese Herbal Medicine, College of Pharmacy, Taipei Medical University, Taipei 11031, Taiwan
6 Departement of Pharmacy, University of Muhammadiyah Mataram, Mataram 83127, Indonesia
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8 Department of Pharmacology, College of Medicine, National Cheng Kung University, Tainan 701, Taiwan
9 Genomics Research Center, Academia Sinica, Taipei 115, Taiwan
10 Institute of Clinical Medicine, National Yang-Ming University, Taipei 112, Taiwan
11 Integrative Therapy Center for Gastroenterologic Cancers, Wan Fang Hospital, Taipei Medical University, Taipei 116, Taiwan
12 Department of Medical Research, Shuang Ho Hospital, Taipei Medical University, New Taipei City 23561, Taiwan
* Correspondence: bkchen58@mail.ncku.edu.tw (B.-K.C.); hiyang@gate.sinica.edu.tw (H.-I.Y.); wcc@tmu.edu.tw (W.-C.C.)

Received: 28 October 2020; Accepted: 6 November 2020; Published: 9 November 2020

Simple Summary: In this study, we systematically evaluated the genetic susceptibility between the store-operated calcium (SOC) influx pathway (stromal interaction molecule 1 (STIM1) and ORAI1) and human hepatocellular carcinoma (HCC) in patients with chronic hepatitis B (CHB) infection. In total, 3631 patients with CHB were recruited, forty polymorphisms of *STIM1* and *ORAI1* were comprehensively analyzed. Three single-nucleotide polymorphisms (SNPs) of *STIM1* (rs6578418, rs11030472, and rs7116520) and one SNP of *ORAI1* (rs6486795) showed a trend of being significantly associated with HCC. In particular, our functional studies (images from total internal reflection fluorescence microscopy and transwell migration assay) revealed that calcium (Ca^{2+}) signaling is essential for the migration of HCC. Based on such a comprehensively screening in 3631 patients with chronic hepatitis, our results indicate the important role of store-operated calcium pathways in HCC.

Abstract: Hepatocellular carcinoma (HCC) often develops from chronic hepatitis B (CHB) through replication of hepatitis B virus (HBV) infection. Calcium (Ca^{2+}) signaling plays an essential role in HBV replication. Store-operated calcium (SOC) channels are a major pathway of Ca^{2+} entry into non-excitable cells such as immune cells and cancer cells. The basic components of SOC signaling

include the *STIM1* and *ORAI1* genes. However, the roles of *STIM1* and *ORAI1* in HBV-mediated HCC are still unclear. Thus, long-term follow-up of HBV cohort was carried out in this study. This study recruited 3631 patients with chronic hepatitis (345 patients with HCC, 3286 patients without HCC) in a Taiwanese population. Genetic variants of the *STIM1* and *ORAI1* genes were detected using an Axiom CHB1 genome-wide array. Clinical associations of 40 polymorphisms were analyzed. Three of the *STIM1* single-nucleotide polymorphisms (SNPs) (rs6578418, rs7116520, and rs11030472) and one SNP of *ORAI1* (rs6486795) showed a trend of being associated with HCC disease ($p < 0.05$). However, after correction for multiple testing, none of the SNPs reached a significant level ($q > 0.05$); in contrast, neither *STIM1* nor *ORAI1* showed a significant association with HCC progression in CHB patients. Functional studies by both total internal reflection fluorescence images and transwell migration assay indicated the critical roles of SOC-mediated signaling in HCC migration. In conclusion, we reported a weak correlation between *STIM1/ORAI1* polymorphisms and the risk of HCC progression in CHB patients.

Keywords: chronic hepatitis B (CHB); hepatocellular carcinoma (HCC); hepatitis B virus (HBV); *STIM1*; *ORAI1*; single-nucleotide polymorphism (SNP)

1. Introduction

Hepatitis B virus (HBV) infection remains the most common chronic viral infection in the world. Despite the development of a vaccine, HBV remains a serious problem worldwide. The World Health Organization (WHO) reported around 257 million new cases and 887,000 deaths due to HBV infection in 2018 [1]. Among them, most patients died from HBV-related complications, including liver cirrhosis and hepatocellular carcinoma (HCC) [2]. HCC, a primary liver cancer, is the fifth most often diagnosed cancer and the second cause of cancer-related deaths among all cancers worldwide [3]. It was also recorded as the second leading cause of cancer-related deaths in East Asia and sub-Saharan Africa and the sixth most common cancer in western countries [4,5]. The incidence of HCC is heterogeneously distributed geographically, with more than 80% of HCC cases occurring in East Asia and sub-Saharan Africa, and China alone accounting for over 50% of global HCC cases [6].

Many factors may contribute to the development of HCC from HBV infection, including host factors (such as a male gender, an older age, being seropositive of the hepatitis B surface antigen (HBsAg), and genetic variants), environmental factors (such as aflatoxin B1 and alcohol consumption), and viral HBV genotypes [6–9]. Moreover, host genetic polymorphisms of several genes, as reported in Asian studies, could be associated with clinical outcomes of HBV-related HCC, including kinesin family member 1b (*KIF1B*) rs17401966 [10], major histocompatibility complex, class II, DQ alpha 1 (*HLA-DQA1*) rs9272105, glutamate ionotropic receptor kainate type subunit 1 (*GRIK1*) rs455804 [11], major histocompatibility complex, class II, DQ alpha 2 (*HLA-DQA2*) rs9275319, signal transducer and activator of transcription 4 (*STAT4*) rs7574865 [12], and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic (*PIK3CA*) rs17849071 [13].

The HBV X protein (HBx), the smallest HBV protein, was reported to mediate the viral replication process in hepatocytes through elevating the intracellular calcium (Ca^{2+}) concentration [14]. Elevation of intracellular Ca^{2+} results from Ca^{2+} release from the endoplasmic reticulum (ER), a major intracellular Ca^{2+} store, and Ca^{2+} influx through Ca^{2+} channels [15,16]. There are two main proteins involved in Ca2+ elevation in unexcitable cells: stromal interaction molecule 1 (STIM1) located in the ER and ORAI1 expressed by plasma membranes [17]. STIM1, a protein involved in the store-operated Ca^{2+} (SOC) entry process, was confirmed to be required by cluster of differentiation 4-positive ($CD4^+$) and cluster of differentiation 8-positive (CD8+) T-cell antiviral immunity [18]. STIM1 acts as a Ca^{2+} sensor, which is able to interact with ORAI1 to trigger Ca^{2+} influx [17]. Previous studies have reported that defective regulation of SOC signaling contributes to the pathogenesis of several diseases including

cancer progression, infections, allergies, and hemostasis [17]. However, the roles of *STIM1* and *ORAI1* in HBV-mediated HCC remain unclear. Herein, we conducted a genetic association study to address this question.

2. Materials and Methods

2.1. Study Subjects

This study is part of the Risk Evaluation of Viral Load Elevation and Associated Liver Disease/Cancer-Hepatitis B Virus (REVEAL-HBV) study, which is a community-based cohort study in Taiwan of individuals infected with HBV aged 30–65 years. The workflow of the study is depicted in Figure 1. In total, 3631 HBV patients (345 patients with HCC and 3286 patients without HCC) met the inclusion criteria (i.e., seronegative for antibodies against the hepatitis C virus/anti-HCV, adequate serum sample of hepatitis B early antigen (HBeAg), seropositive for the hepatitis B surface antigen (HBsAg), and serum levels of alanine aminotransferase (ALT) and HCC-free patients at study entry) [19]. The period of study recruitment was 1991–1992 with a follow-up until 2014 in seven regions of Taiwan (i.e., Sanchi, Chutung, Potzu, Kaoshu, Makung, Hushi, and Paisha). All of the study participants were ethnic Chinese (i.e., Taiwanese). They provided written informed consent before participation as a declaration, agreeing that we could conduct an interview, collect a blood specimen, and conduct various serologic and biochemical assays. This project was approved by the ethics committees at Academia Sinica (AS-IRB-BM-15017) Taipei, Taiwan. All participants were interviewed in person using a structured questionnaire administered by well-trained public health nurses. Sociodemographic characteristics of each patient were queried, including their medical and surgical histories, and any family history of HCC.

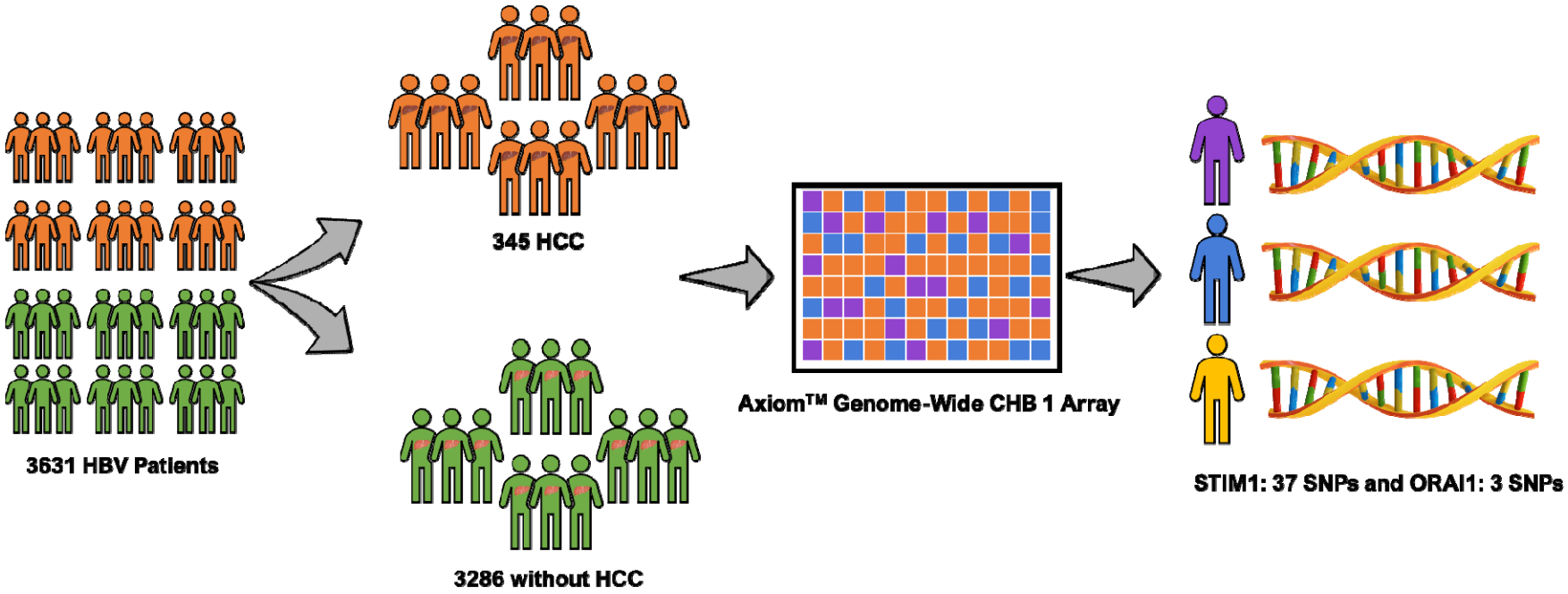

Figure 1. Data from 3631 hepatitis B virus (HBV) patients including 3286 subjects without hepatocellular carcinoma (HCC) and 345 subjects with HCC were analyzed. Axiom-chronic hepatitis B -(CHB) genome-wide array was used for genotyping. A total of 40 single nucleotide polymorphism (SNPs) were assessed (37 *STIM1* and three *ORAI1*) through this method.

2.2. Clinical Evaluation

All participants in the REVEAL-HBV study were followed-up every 6–12 months to check for a clinical evaluation of HBV characteristics including determining the HBsAg level, the serostatus of HBsAg, the serostatus of HBeAg, and the ALT level. The serostatuses of HBsAg, HBeAg, and ALT were tested using commercial kits. Levels of HBsAg and HBeAg were quantified with a radioimmunoassay (Abbott Laboratories, North Chicago, IL, USA). Serum HBsAg levels were quantified using a Roche Elecsys HBsAg II Quant assay. ALT was quantified using a serum chemistry autoanalyzer (model 736; Hitachi, Tokyo, Japan) with commercial reagents. The HBV genotype was determined in those with detectable serum levels of HBV DNA by a melting curve analysis.

2.3. Ascertainment of HCC

All participants of this study were ascertained to not have HCC at the time of study entry, and cases of HCC during follow-up were determined by computerized linkage of data with information from the National Cancer Registry in Taiwan. The HCC diagnosis was based on pathological examination of hepatic specimens, positive lesions with confirmation, which included at least two different imaging techniques (angiogram, computed tomography, or abdominal ultrasonography), or positive lesions by one imaging technique accompanied by an α-fetoprotein level of $\geq$400 ng/mL.

2.4. Genotyping of SNPs

Our study is part of the REVEAL-HBV cohort study, a community-based cohort (consisting of 345 patients with HCC during follow-up and 3286 patients without HCC). Human genomic DNA was extracted from peripheral blood leukocytes using Qiagen commercial kits (QIAamp DNA Blood Maxi Kit) and standard methods. Axiom CHB1 genome-wide array was applied to discover genes potentially associated with HBV-related HCC.

2.5. SNP Annotation Data Query

In order to evaluate the relationship between SNPs and profiles of gene expression, we confirmed it by examining the expression quantitative trait loci (e-QTL) through Genotype-Tissue Expression (GTEx) portal database (http://www.gtexportal.org/home/), which contains the expressions of genes in a variety of tissues.

2.6. Cell Culture

The liver cancer cell lines Huh 7 and HepG2 were cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen, 32571036) and incubated at 37 °C with 5% CO_2 for the complete culture medium, and the DMEM was contained with 100 mg/mL streptomycin, 100 U/mL penicillin (Invitrogen, 15140122) and 10% (vol/vol) bovine calf serum (Invitrogen, 10437-028).

2.7. Plasmids Transfection

The plasmids STIM1-yellow fluorescent protein (STIM1-YFP) (Addgene, #19754) and Orai1-monomeric red fluorescent proteins (Orai1-mCherry) were used in real-time total internal reflection fluorescence microscope (TIRF) imaging systems. For expression of the plasmids in cells, the plasmids were transfected with TurboFect (Thermo Fisher Scientific, R0532) for 24 h. The cells were re-seeded on Lab-Tek chambered cover glass (Thermo Fisher Scientific, NUC155383) before observed with a microscope.

2.8. Transwell Migration Assay

To examine the effect of SOC inhibitor, 2-aminoethoxydiphenylborate (2-APB) on cell motility, the Huh 7 or HepG2 cells were pre-treated with 2-APB for 30 minutes. The 1×10^5 cells were seeded in the transwell insert (BD, 353097) contained with serum-free medium and the complete medium

was added into the 24-well plate as a chemo-attractant. After 24 or 48 hours, the inserts were washed with phosphate-buffered saline (PBS) and the cells in the inserts were removed. The cells attached to the reverse side of the insert were fixed with ice cold methional and stained with crystal violet. The migrated cells were photographed by an inverted microscope (LEICA, DM IL LED) with 20× objective lens and analyzed with Image J software.

2.9. Total Internal Reflection Fluorescence Microscopy (TIRF)

In order to observe the effects of 2-APB on thapsigargin (TG)-induced SOC activity, the STIM1-YFP and Orai1-mCherry co-expressed Huh 7 cells were subcultured on Lab-Tek chambered cover glass before imaging was taken by a microscope. The total internal reflection fluorescence microscope (TIRFM) system was built on an inverted microscope (Olympus IX81, Tokyo, Japan) with a high-sensitivity Electron Multiplying Charge Coupled Device (EMCCD) camera (iXOn3897, Andor Technology, Tubney Woods, Abingdon, UK). In order to observe the fluorescent signal on the cell membrane, the UPONAPO 60x OTIRF objective lens (NA: 1.49; Olympus) was applied to achieve TIRF images. The fluorescent proteins labeled with YFP and mCherry were excited with 488 and 532 nm solid lasers, respectively, and the program was driven by Xcellence software (Olympus imaging software).

2.10. Statistical Analysis

Distributions of single-nucleotide polymorphisms (SNPs) of *STIM1* and *ORAI1* were tested for Hardy–Weinberg equilibrium (HWE) in order to evaluate allelic distributions between the two populations. All SNPs in our study met the HWE condition (all $p > 0.05$) with minor allele frequency (MAF) of >5%. Linkage disequilibrium (LD) was evaluated with Haploview software version 4.2 (Broad Institute, Cambridge, MA, USA). LD was examined for SNP pairs, and haplotype blocks were defined using the default setting of the Haploview software version 4.2. We used logistic regression analyses to obtain odds ratio (OR) of HCC cases adjusted using age, gender, ALT, HBsAg level, HBeAg serostatus, and viral genotype. Associations between SNPs and HCC under the genotype, dominant, and allelic models were determined using the R "SNPassoc" package analyses in the R environment (https://cran.r-project.org/ and https://www.r-project.org/), multiple testing correction was performed using the false discovery rate (FDR), and q-values of <0.05 were determined to indicate statistical significance. Haplotype associations with HCC were determined with Haploview software version 4.2.

3. Results

3.1. Basic Characteristics of Chronic Hepatitis B-Infected Patients

We analyzed the data from 3631 chronic HBV-infected subjects, which included 345 chronic hepatitis B (CHB) patients with HCC progression and 3286 CHB patients without HCC progression. ALT, the HBsAg levels, and the HBeAg serostatus were monitored, and the mean and range of age, gender, and viral genotype are shown in Table 1. Results indicated that the average age of CHB patients was 45.7 years, and male gender was more dominant (2196 subjects) compared to female gender (1435 subjects). Serological marker of ALT level indicated that patients with an ALT level of <45 U/L were more dominant (3405 subjects) compared to patients with an ALT level of >45 U/L (226 subjects). In case of the serological marker of HBsAg level, there are 1519 subjects with a HBsAg level >1000 IU/mL, 946 subjects with a HBsAg level <1000 IU/mL and 918 subjects with a HBsAg level <100 IU/mL. Multivariate analyses showed that participants of an older age had a significantly higher risk of suffering from HCC, with an OR of 2.32 for those 40–49 years, an OR of 3.47 for those 50–59 years, and an OR of 3.8 for those >60 years after adjusting for gender and serum ALT. Male patients had a 2.53-fold tendency of a higher risk compared to female CHB patients after adjusting for age and serum ALT. Patients with an ALT level of >45 U/L had a 3.7-fold tendency of a higher risk compared to CHB patients with an ALT level of <45 U/L after adjusting for age and gender.

Table 1. Baseline characteristics and multivariate adjusted odd ratios (ORs) of hepatocellular carcinoma (HCC) according to various risk factors.

Characteristic	Overall *N* = 3631 (%)	Without HCC (*N* = 3286)	With HCC (*N* = 345)	OR (95% CI)	*p* Value
Age, mean years (SD)	45.77	45.32 (9.65)	50.08 (8.87)	-	
Age range (years)	30~65	30~65	30~65	-	
30~39	1194 (32.88)	1140 (34.69)	54 (15.65)	Ref	
40~49	1031 (28.39)	931 (28.33)	100 (28.98)	2.32 (1.64~3.29)	**<0.001** *
50~59	1049 (28.90)	911 (27.72)	138 (40.00)	3.47 (2.48~4.84)	**<0.001** *
≥60	357 (9.83)	304 (9.25)	53 (15.36)	3.8 (2.53~5.72)	**<0.001** *
Gender					
Female, *n* (%)	1435 (39.52)	1365 (41.54)	70 (20.29)	Ref	
Male, *n* (%)	2196 (60.48)	1921 (58.46)	275 (79.71)	2.53 (1.86~3.45)	**<0.001** *
ALT (U/L)					
<45	3405 (93.78)	3118 (94.89)	287 (83.18)	Ref	
≥45	226 (6.22)	168 (5.12)	58 (16.82)	3.7 (2.65~5.17)	**<0.001** *
HBsAg level (IU/mL)£					
<100	918 (27.14)	880 (28.59)	38 (12.46)	Ref	
≤1000	946 (27.96)	883 (28.69)	63 (20.66)	2.01 (1.32~3.07)	**0.001** *
≥1000	1519 (44.90)	1315 (42.72)	204 (66.89)	5.24 (3.6~7.63)	**<0.001** *
HBeAg serostatus †					
Negative	2962 (84.70)	2765 (87.58)	197 (57.94)	Ref	
Positive	535 (15.30)	392 (12.42)	143 (42.06)	7.87 (5.95~10.42)	**<0.001** *
Viral genotype ††					
B+(BC)	1734 (65.28)	1607 (68.04)	127 (43.19)	Ref	
C	922 (34.71)	755 (31.96)	167 (56.80)	3.06 (2.36,3.97)	**<0.001** *

SD, standard deviation; ALT, alanine aminotransferase; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B early antigen. * Significant *p* value (<0.05) are in **bold**. £ Data were only available for 3383 samples. † Data were only available among the Risk Evaluation of Viral Load Elevation and Associated Liver Disease/Cancer-Hepatitis B Virus (REVEAL-HBV) cohort for 3497 samples. †† Data were available for 2656 individuals with detectable HBV DNA levels (≥300 copies/mL).

Patients with a HBsAg level of >1000 IU/mL had a 5.24-fold higher risk of suffering from HCC compared to those with a HBsAg level of <1000 IU/mL after adjusting for age, ALT, and gender. Subjects who were HBeAg positive also had a 7.87-fold higher risk of HCC compared to those who were negative for HBeAg after adjusting for age, ALT, and gender. Subjects with the viral genotype type C had a 3.06-fold higher risk of suffering from HCC compared to those with genotype B or B+C after adjusting for age, ALT, and gender (Table 1).

3.2. *STIM1 and ORAI1 SNPs with Minor Allelic Frequencies in Different Populations*

Table 2 shows the MAFs of *STIM1* and *ORAI1* polymorphisms in different populations worldwide (e.g., Asian, African, American, European, and Taiwanese). MAFs of African, American, European, and Asian populations were extracted from the HaploReg browser version 4.1 (https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php), and MAFs of the Taiwan Biobank (TWB) were obtained from the Taiwan View website (https://taiwanview.twbiobank.org.tw/index). In addition, allele frequencies in the current study are similar to those in reference to Asians, including the TWB. All SNPs met the HWE condition with an MAF of >5%.

Table 2. Minor allelic frequencies of single-nucleotide polymorphisms (SNPs) in this study.

SNP	Position (hg38) (bp)	Gene	Allele		Minor Allelic Frequencies (MAFs)						
			Major	Minor	AFR	AMR	ASN	EUR	TWB	Ours	HWE
rs4243966	Chr11:3864993	*STIM1*	T	C	0.05	0.07	0.04	0.06	0.04	0.04	0.95
rs7943201	Chr11:3865531	*STIM1*	G	A	0.33	0.45	0.36	0.37	0.38	0.37	0.62
rs11030122	Chr11:3865946	*STIM1*	C	G	0.09	0.23	0.34	0.33	0.34	0.34	0.65
rs7951076	Chr11:3866612	*STIM1*	G	A	0.11	0.31	0.28	0.29	0.25	0.26	0.74
rs7952083	Chr11:3869730	*STIM1*	A	C	0.19	0.41	0.38	0.49	0.38	0.39	0.60
rs7120828	Chr11:3879685	*STIM1*	C	T	0.48	0.26	0.34	0.22	0.35	0.34	0.66
rs10458894	Chr11:3892042	*STIM1*	C	T	0.23	0.39	0.31	0.35	0.30	0.31	0.68
rs7120683	Chr11:3894732	*STIM1*	T	G	0.43	0.27	0.34	0.22	0.35	0.34	0.65
rs10835262	Chr11:3895276	*STIM1*	G	A	0.19	0.23	0.34	0.34	0.34	0.34	0.66

Table 2. *Cont.*

SNP	Position (hg38) (bp)	Gene	Allele		Minor Allelic Frequencies (MAFs)						
			Major	Minor	AFR	AMR	ASN	EUR	TWB	Ours	HWE
rs4622250	Chr11:3897371	*STIM1*	C	T	0.09	0.22	0.30	0.33	0.25	0.25	0.75
rs75197750	Chr11:3901583	*STIM1*	C	T	0.03	0.04	0.05	0.03	0.03	0.05	0.98
rs10500589	Chr11:3902756	*STIM1*	T	C	0.02	0.23	0.01	0.02	0.21	0.22	0.77
rs11030209	Chr11:3903119	*STIM1*	A	C	0.21	0.23	0.29	0.33	0.25	0.25	0.74
rs10835270	Chr11:3903372	*STIM1*	G	T	0.31	0.28	0.31	0.38	0.27	0.27	0.72
rs11030210	Chr11:3903482	*STIM1*	G	T	0.01	0.07	0.03	0.06	0.04	0.04	0.95
rs7929653	Chr11:3904546	*STIM1*	G	A	0.31	0.46	0.40	0.43	0.43	0.44	0.56
rs6578418	Chr11:3905002	*STIM1*	C	G	0.08	0.09	0.08	0.08	0.08	0.07	0.93
rs10835272	Chr11:3905008	*STIM1*	G	A	0.43	0.26	0.47	0.14	0.46	0.45	0.55
rs10742189	Chr11:3907522	*STIM1*	A	C	0.33	0.37	0.43	0.49	0.39	0.39	0.60
rs7129444	Chr11:3911802	*STIM1*	C	T	0.36	0.29	0.35	0.42	0.31	0.32	0.67
rs7118422	Chr11:3912065	*STIM1*	C	T	0.22	0.48	0.48	0.48	0.46	0.45	0.55
rs4910863	Chr11:3917892	*STIM1*	T	C	0.08	0.46	0.46	0.47	0.44	0.43	0.56
rs11030264	Chr11:3919001	*STIM1*	A	G	0.04	0.49	0.43	0.46	0.41	0.41	0.58
rs7924984	Chr11:3953358	*STIM1*	G	A	0.30	0.33	0.46	0.35	0.47	0.47	0.52
rs2412338	Chr11:3956291	*STIM1*	G	T	0.31	0.33	0.46	0.35	0.47	0.48	0.51
rs10835402	Chr11:3969684	*STIM1*	T	C	0.02	0.24	0.30	0.32	0.28	0.28	0.72
rs11030472	Chr11:3978105	*STIM1*	A	G	0.00	0.00	0.16	0.00	0.18	0.19	0.80
rs11030478	Chr11:3979345	*STIM1*	G	A	0.11	0.08	0.15	0.02	0.20	0.20	0.79
rs11030486	Chr11:3980034	*STIM1*	C	T	0.11	0.08	0.15	0.02	0.20	0.20	0.79
rs12284835	Chr11:3997798	*STIM1*	G	A	0.08	0.03	0.05	0.08	0.04	0.05	0.95
rs727152	Chr11:3998535	*STIM1*	A	G	0.50	0.12	0.10	0.16	0.10	0.10	0.89
rs2959081	Chr11:4005810	*STIM1*	T	C	0.12	0.48	0.44	0.49	0.41	0.40	0.60
rs11030639	Chr11:4017826	*STIM1*	A	G	0.30	0.26	0.32	0.33	0.31	0.31	0.68
rs7116520	Chr11:4032147	*STIM1*	A	G	0.10	0.46	0.47	0.45	0.42	0.42	0.58
rs1442725	Chr11:4068357	*STIM1*	C	T	0.48	0.10	0.05	0.08	0.05	0.05	0.94
rs11030841	Chr11:4077783	*STIM1*	G	A	0.16	0.22	0.21	0.12	0.26	0.26	0.73
rs4910882	Chr11:4081185	*STIM1*	G	A	0.16	0.22	0.21	0.12	0.26	0.26	0.73
rs6486795	Chr12:121638011	*ORAI1*	T	C	0.36	0.28	0.38	0.21	0.38	0.37	0.62
rs74936888	Chr12:121638300	*ORAI1*	T	C	0.07	0.00	0.13	0.01	0.08	0.08	0.92
rs3741595	Chr12:121641283	*ORAI1*	T	C	0.07	0.24	0.21	0.15	0.26	0.26	0.73

AFR, African; AMR, American; ASN, Asian; EUR, European; TWB, Taiwan Biobank; HWE, Hardy–Weinberg equilibrium. Minor allele frequencies (MAFs) of AFR, AMR, ASN and EUR were extracted from the HaploReg browser version 4.1 (https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php) and MAFs of the TWB were obtained from the Taiwan View website (https://taiwanview.twbiobank.org.tw/index).

3.3. Genetic Association of *STIM1* and *ORAI1* with HCC

Our study assessed 37 *STIM1* polymorphisms and three *ORAI1* polymorphisms (Table 3). Three genetic models (allelic, genotype, and dominant) were applied to test the association between HCC and these SNPs. rs6578418, rs11030472, and rs7116520 of *STIM1* and rs6486795 of *ORAI1* showed a trend of being significantly associated with HCC disease ($p < 0.05$). However, after correction for multiple testing, none of the SNPs reached a significant level ($q > 0.05$) (Table 3).

Table 3. Association of *STIM1* and *ORAI1* genes with hepatocellular carcinoma in hepatitis B virus (HBV) patients under allelic, genotypic, and dominant models.

SNP	Gene	Effect Allele	(Allelic) *p* Value [a] [*q* Value]	(Genotype) *p* Value [b] [*q* Value]	(Dominant) *p* Value [b] [*q* Value]
rs4243966	*STIM1*	C	0.790 [0.922]	0.602 [0.900]	0.630 [0.968]
rs7943201	*STIM1*	A	0.922 [0.922]	0.881 [0.943]	0.901 [0.989]
rs11030122	*STIM1*	G	0.580 [0.922]	0.804 [0.900]	0.970 [0.989]
rs7951076	*STIM1*	A	0.380 [0.922]	0.811 [0.900]	0.527 [0.867]
rs7952083	*STIM1*	C	0.449 [0.922]	0.581 [0.900]	0.865 [0.989]
rs7120828	*STIM1*	T	0.889 [0.922]	0.722 [0.900]	0.704 [0.989]
rs10458894	*STIM1*	T	0.382 [0.922]	0.629 [0.900]	0.380 [0.867]
rs7120683	*STIM1*	G	0.504 [0.922]	0.760 [0.900]	0.470 [0.867]

Table 3. *Cont.*

SNP	Gene	Effect Allele	(Allelic) *p* Value [a] [*q* Value]	(Genotype) *p* Value [b] [*q* Value]	(Dominant) *p* Value [b] [*q* Value]
rs10835262	*STIM1*	A	0.788 [0.922]	0.972 [0.972]	0.989 [0.989]
rs4622250	*STIM1*	T	0.655 [0.922]	0.626 [0.900]	0.876 [0.989]
rs75197750	*STIM1*	T	0.354 [0.922]	0.383 [0.900]	0.321 [0.867]
rs10500589	*STIM1*	C	0.428 [0.922]	0.707 [0.900]	0.405 [0.867]
rs11030209	*STIM1*	C	0.651 [0.922]	0.765 [0.900]	0.823 [0.989]
rs10835270	*STIM1*	T	0.274 [0.922]	0.438 [0.900]	0.199 [0.858]
rs11030210	*STIM1*	T	0.860 [0.922]	0.353 [0.900]	0.781 [0.989]
rs7929653	*STIM1*	A	0.656 [0.922]	0.622 [0.900]	0.980 [0.989]
rs6578418	*STIM1*	G	**0.048** [0.922]	0.133 [0.900]	**0.044** [0.527]
rs10835272	*STIM1*	A	0.351 [0.922]	0.241 [0.900]	0.978 [0.989]
rs10742189	*STIM1*	C	0.351 [0.922]	0.241 [0.900]	0.978 [0.989]
rs7129444	*STIM1*	C	0.588 [0.922]	0.816 [0.900]	0.544 [0.867]
rs7118422	*STIM1*	T	0.542 [0.9222]	0.899 [0.943]	0.734 [0.989]
rs4910863	*STIM1*	C	0.634 [0.922]	0.735 [0.900]	0.486 [0.867]
rs11030264	*STIM1*	G	0.697 [0.922]	0.954 [0.972]	0.788 [0.989]
rs7924984	*STIM1*	A	0.450 [0.922]	0.530 [0.900]	0.284 [0.867]
rs2412338	*STIM1*	T	0.382 [0.922]	0.568 [0.900]	0.293 [0.867]
rs10835402	*STIM1*	C	0.292 [0.922]	0.487 [0.900]	0.370 [0.867]
rs11030472	*STIM1*	G	0.074 [0.922]	0.068 [0.900]	**0.029** [0.527]
rs11030478	*STIM1*	A	0.810 [0.922]	0.689 [0.900]	0.859 [0.989]
rs11030486	*STIM1*	T	0.765 [0.922]	0.698 [0.900]	0.806 [0.989]
rs12284835	*STIM1*	A	0.129 [0.922]	0.118 [0.900]	0.110 [0.594]
rs727152	*STIM1*	G	0.339 [0.922]	0.671 [0.900]	0.402 [0.867]
rs2959081	*STIM1*	C	0.138 [0.922]	0.140 [0.900]	0.061 [0.527]
rs11030639	*STIM1*	G	0.132 [0.922]	0.181 [0.900]	0.317 [0.867]
rs7116520	*STIM1*	G	**0.016** [0.922]	**0.033** [0.900]	**0.009** [0.399]
rs1442725	*STIM1*	T	0.204 [0.922]	0.287 [0.900]	0.259 [0.867]
rs11030841	*STIM1*	A	0.140 [0.922]	0.367 [0.900]	0.484 [0.867]
rs4910882	*STIM1*	A	0.156 [0.922]	0.392 [0.900]	0.532 [0.867]
rs6486795	*ORAI1*	C	**0.013** [0.594]	0.129 [0.900]	0.055 [0.527]
rs74936888	*ORAI1*	C	0.050 [0.922]	0.207 [0.900]	0.091 [0.594]
rs3741595	*ORAI1*	C	0.074 [0.922]	0.294 [0.900]	0.195 [0.858]

[a] Fisher's exact *p* value. [b] Adjusted by alanine transaminase, gender, and age. Significant *p* values are in **bold**. The *q* value is the false discovery rate (FDR) estimation for multi-testing.

For *STIM1*, rs6578418 showed a weak association under the allelic model ($p = 0.048$) and dominant model ($p = 0.044$). In addition, rs11030472 showed a weak association under the dominant model ($p = 0.0296$), and rs7116520 showed a weak association with HCC under the genotypic ($p = 0.0330$), dominant ($p = 0.0093$), and allelic models ($p = 0.0165$). In *ORAI1*, only rs6486795 among the three

SNPs that we evaluated showed a trend of being associated with HCC after adjusting for serum ALT, gender, and age. rs6486795 showed a weak association under the allelic model, with $p = 0.0135$. However, after correction for multiple testing, none of the SNPs reached a significant level (Table 3).

3.4. Haplotype Analysis of STIM1 and ORAI1

We further performed haplotype analysis. Results showed weak correlations of *STIM1* (Figure S1) and *ORAI1* (Figure S2) haplotypes with HCC disease. For *STIM1*, both GGTAGCAT haplotype of block six and AGCGG haplotype of block seven had trends of being associated with HCC at $p = 0.0163$ and $p = 0.015$, respectively (Table S1). In addition, results for *ORAI1* indicated a trend of HBV carriers with the TTC ($p = 0.013$) haplotype being more susceptible to associate with HCC progression than those with the CTT, CCC, or CTC haplotype (Table S2). Unfortunately, none of these haplotypes of *STIM1* and *ORAI1* were still significant after applying the multiple correction.

3.5. Annotation of Expression Quantitative Trait Loci (e-QTLs) for STIM1 and ORAI1

To elaborate the relationship between the genetic polymorphisms and gene expression, the publicly available database GTEx portal (http://www.gtexportal.org/home/) was utilized to study the tissue expression quantitative trait loci. For rs6578418 and rs11030472, we could not find the gene expression data on the GTEx portal database due to limited available tissues (Table S3). While the trend of *STIM1* rs7116520 showed that GG genotype had a higher *STIM1* expression compared to those with AG and AA genotypes (Figure S3). Furthermore, *ORAI1* rs6486795 with CC genotype was associated with higher *ORAI1* expression compared to those with TC and TT genotypes in a variety of tissues (e.g., whole blood, esophagus, thyroid, heart, stomach, pancreas, muscle, colon).

3.6. 2-APB Inhibited Liver Cancer Cell Migration via Suppressed STIM1-ORAI1 Colocolization

STIM1 and ORAI1 are two basic components of SOC as a major pathway of calcium entry in non-excitable cells, especially in the cancer cells [17]. To validate the roles of SOC influx pathway (STIM1 and ORAI1) in HCC cell progression, 2-APB, a SOC inhibitor was applied. As shown in Figure 2A, the colocalization of STIM1 and ORAI1 was inhibited by 2-APB pre-treatment for 30 minutes in Huh 7 cells. We further analyzed the inhibitory effects of 2-APB in cell migration by using Huh 7 and HepG2 cell lines. Results showed that liver cancer cells migration (Huh 7 and HepG2) significantly decreased by 2-APB pre-treatment compared to the control group (Figure 2B,C).

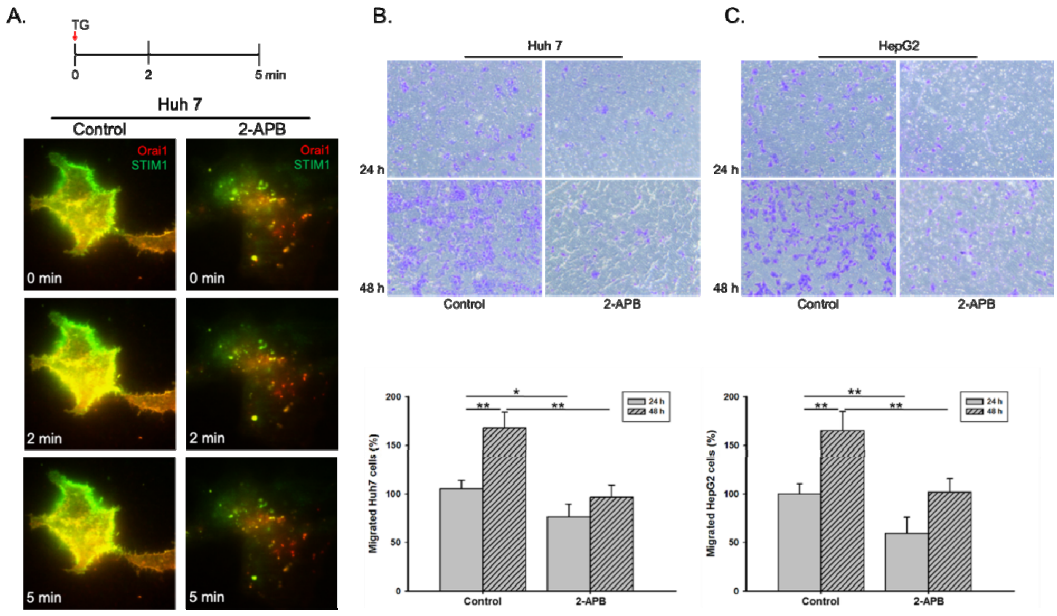

Figure 2. Inhibition of STIM1-ORAI1 colocalization by 2-APB pre-treatment leads to reduced liver

cancer cell migration ability. The Huh 7 cells were pre-treated with store-operated calcium (SOC) inhibitor, 2-APB, for 30 min. (**A**). The cells were co-transfected with STIM1-YFP and Orai1-mCherry and reseeded on Lab-Tek chambered cover glass for 24 h. The inhibition effect of 2-APB on thapsigargin (TG)-induced SOC activity was observed with time-lapse TIRFM images. The inhibition effect of 2-APB on (**B**) Huh 7 or (**C**) HepG2 cell migration ability was examined by transwell migration assay. Statistically significant data are indicated by * for $p < 0.05$ and ** for $p < 0.01$.

4. Discussion

Hepatocellular carcinoma (HCC) is the second highest cause of cancer-related deaths worldwide [3]. Despite the fact that CHB is a major cause of HCC [2], only a fraction of CHB-infected patients develops HCC during their lifetime. Therefore, it is important to determine the potential protective or risk factors related to HCC susceptibility. Our investigation comprised a cohort study with 3631 CHB patients consisting of 345 CHB patients with HCC progression and 3286 CHB patients without HCC progression. This study was part of the REVEAL-HBV cohort study, which previously revealed that male patients, older patients and those with a higher ALT level, with HBeAg positive or who had viral genotype C had a significantly higher risk of CHB-related HCC progression [8,19,20]. Calcium signaling has been shown as a critical factor for viral replication during HBV pathogenesis [14]. Mitochondrial Ca^{2+} uptake, SOC pathway and the C-terminal of ORAI1 protein involved in the regulatory mechanism of HBX protein-modulated cytosolic calcium increase [15,21,22]. In this study, we systematically evaluated the genetic susceptibility between the SOC influx pathway (STIM1 and ORAI1) and human HCC in patients with CHB infection. In total, 3631 patients with chronic hepatitis were recruited. Forty polymorphisms in the SOC pathway were comprehensively analyzed. Three SNPs of *STIM1* (rs6578418, rs11030472, and rs7116520) and one SNP of *ORAI1* (rs6486795) showed a trend of correlation with HCC. However, none of these SNPs reached the significance after multiple correction. Interestingly, the trend of risk alleles of four variants (rs6578418, rs11030472, rs7116520, and rs6486795) showed higher frequencies in Asian populations compared to European and American populations. The trend of the G allele as a risk allele of rs11030472 represented 16–19% frequencies, in current study (19%), in the TWB (18%) and in the Asian region (16%), compared to European, African, and American at 0%. In addition, variant rs6486795 of the C allele showed an allelic frequency in an Asian population of around 38%, and our study also showed a 37% frequency compared to American and European populations at 28% and 21%, respectively. For two variants, the G allele of rs6578418 and rs7116520 exhibited homogeneity in Asian (rs6578418, 8% and rs7116520, 47%), European (rs6578418, 8% and rs7116520, 45%) and American (rs6578418, 9% and rs7116520, 46%) populations, respectively. It is likely that variations in allelic frequencies among different populations may play a role in the observed different results in other populations. However, we cannot rule out the possibility that other low/high frequency genetic polymorphisms of *STIM1* and *ORAI1* may contribute to HCC progression in CHB.

The roles of the *STIM1* and *ORAI1* genes were also determined in other diseases. For example, rs6486795 of the *ORAI1* gene was associated with various diseases, including atopic dermatitis in Japanese and Taiwanese populations [23] and nephrolithiasis in a Taiwanese population [24]. In addition to these results, other studies also reported that polymorphisms of *ORAI1* are involved in other diseases such as ankylosing spondylitis and Kawasaki disease [25,26]. Results of haplotype analyses indicated that HBV carriers with the GGTAGCAT haplotype had a trend of being associated with a HCC risk; additionally, those with the AGCGG haplotype also showed a trend of being more susceptible to HCC progression. Results for *ORAI1* also indicated a trend of HBV carriers with the TTC haplotype being more susceptible to being associated with a HCC risk than those with the CTT, CCC, or CTC haplotype. However, we found no significant association after applying the multiple correction.

Calcium influx through the SOC channel plays a pivotal role in regulating many signaling pathways related to cancer progression, infections, allergies, and hemostasis [17], such as colorectal cancer [27,28], cervical cancer [29] melanomas [30], and lung cancer [31] and breast cancer [32]. Functional studies have indicated the inhibition of *STIM1* and *ORAI1* in the metastasis of cancer cells [32]. Mutations of *STIM1* and *ORAI1* were also reported to affect patients with viral, bacterial,

and fungal infections via abolishing the SOC influx and immune system [33,34]. Thus, the correlations between genetic factors and HCC progression in HBV infection might due to the increase in Ca^{2+} levels that initially commence from SOC influx, which is the predominant mechanism of Ca^{2+} entry in hepatocytes [21]. The HBx protein is the smallest HBV protein that has been reported in mediating HBV replication and development of HCC through elevation of cytosolic calcium signals [21,35]. Therefore, an association study of *STIM1* and *ORAI1* polymorphisms for HBV DNA level was also examined in this study; however, the results did not show a significant association (data not shown). Elevation of Ca^{2+} through the SOC channel has an important role in hepatocytes. Indeed, the elevation of cytosolic calcium involves the replication of the HBV and hepatitis C virus (HCV), which might cause HCC [15,36]. Here, our study also indicated that knockdown of the SOC pathway by 2-APB prevented cell migration in two liver cancer cell lines (Huh 7 and HepG2). We acknowledge that the weak correlation between *STIM1* and *ORAI1* polymorphisms and the risk of HCC progression in CHB patients may be due to the modest sample size (3631 CHB patients), which led to the reduced power of the statistical analysis. Another possibility is that the majority of polymorphisms were located in the non-coding region. The genetic effects of each polymorphism for the expression of the store-operated calcium channel are mild.

5. Conclusions

Our study indicated that inhibition of the SOC pathway is able to block cell migration in two liver cancer cell lines (Huh 7 and HepG2). Although genetic polymorphisms of the SOC pathway (*STIM1* and *ORAI1*) are not significantly associated with HCC progression after multiple correction, three SNPs of *STIM1* (rs6578418, rs11030472, and rs7116520) and one SNP of *ORAI1* (rs6486795) showed a borderline significant trend that should be particularly focused on in future studies.

Supplementary Materials: The following are available online at http://www.mdpi.com/2079-7737/9/11/388/s1. Table S1: Association between *STIM1* haplotypes and hepatocellular carcinoma (HCC) in hepatitis B virus (HBV) patients. Table S2: Association between *ORAI1* haplotypes and hepatocellular carcinoma (HCC) in hepatitis B virus (HBV) patients. Table S3. Expression quantitative trail loci (eQTL) results of the SNP from Genotype-tissue expression (GTEx). Supplementary Figure S1: Linkage disequilibrium plot of the *STIM1* gene. Figure S2: Linkage disequilibrium plot of the *ORAI1* gene. Figure S3: Correlation between genotype and expression in tissue determined by *Cis*-expression quantitative trait loci (*cis*-eQTLs).

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References

1. World Health Organization. Hepatitis B. Available online: https://www.who.int/news-room/fact-sheets/detail/hepatitis-b (accessed on 17 June 2019).
2. Jefferies, M.; Rauff, B.; Rashid, H.; Lam, T.; Rafiq, S. Update on global epidemiology of viral hepatitis and preventive strategies. *World J. Clin. Cases* **2018**, *6*, 589–599. [CrossRef] [PubMed]
3. Forner, A.; Reig, M.; Bruix, J. Hepatocellular carcinoma. *Lancet* **2018**, *391*, 1301–1314. [CrossRef]
4. Bray, F.; Me, J.F.; Soerjomataram, I.; Siegel, R.L.; Torre, L.A.; Jemal, A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA A Cancer J. Clin.* **2018**, *68*, 394–424. [CrossRef] [PubMed]
5. Choo, S.P.; Tan, W.L.; Goh, B.K.P.; Tai, W.M.; Zhu, A.X. Comparison of hepatocellular carcinoma in Eastern versus Western populations. *Cancer* **2016**, *122*, 3430–3446. [CrossRef]

6. El-Serag, H.B. Epidemiology of Viral Hepatitis and Hepatocellular Carcinoma. *Gastroenterol.* **2012**, *142*, 1264–1273.e1. [CrossRef]
7. Bruix, J.; Reig, M.; Sherman, M. Evidence-Based Diagnosis, Staging, and Treatment of Patients with Hepatocellular Carcinoma. *Gastroenterology* **2016**, *150*, 835–853. [CrossRef]
8. Kew, M. Epidemiology of chronic hepatitis B virus infection, hepatocellular carcinoma, and hepatitis B virus-induced hepatocellular carcinoma. *Pathol. Biol.* **2010**, *58*, 273–277. [CrossRef] [PubMed]
9. Burns, G.S.; Thompson, A.J. Viral Hepatitis B: Clinical and Epidemiological Characteristics. *Cold Spring Harb. Perspect. Med.* **2014**, *4*, a024935. [CrossRef]
10. Zhang, H.; Zhai, Y.; Hu, Z.; Wu, C.; Qian, J.; Jia, W.; Ma, F.; Huang, W.; Yu, L.; Yue, W.; et al. Genome-wide association study identifies 1p36.22 as a new susceptibility locus for hepatocellular carcinoma in chronic hepatitis B virus carriers. *Nat. Genet.* **2010**, *42*, 755–758. [CrossRef]
11. Li, S.; Qian, J.; Yang, Y.; Zhao, W.; Dai, J.; Bei, J.-X.; Foo, J.N.; McLaren, P.J.; Li, Z.; Yang, J.; et al. GWAS Identifies Novel Susceptibility Loci on 6p21.32 and 21q21.3 for Hepatocellular Carcinoma in Chronic Hepatitis B Virus Carriers. *PLoS Genet.* **2012**, *8*, e1002791. [CrossRef]
12. Jiang, D.-K.; Sun, J.; Cao, G.; Liu, Y.; Lin, D.; Gao, Y.-Z.; Ren, W.-H.; Long, X.-D.; Zhang, H.; Ma, X.-P.; et al. Genetic variants in STAT4 and HLA-DQ genes confer risk of hepatitis B virus–related hepatocellular carcinoma. *Nat. Genet.* **2012**, *45*, 72–75. [CrossRef] [PubMed]
13. Li, H.-G.; Liu, F.-F.; Zhu, H.-Q.; Zhou, X.; Lu, J.; Chang, H.; Hu, J.-H. PIK3CA polymorphisms associated with susceptibility to hepatocellular carcinoma. *Int. J. Clin. Exp. Pathol.* **2015**, *8*, 15255–15259. [PubMed]
14. Geng, X.; Huang, C.; Qin, Y.; McCombs, J.E.; Yuan, Q.; Harry, B.L.; Palmer, A.E.; Xia, N.S.; Xue, D. Hepatitis B virus X protein targets Bcl-2 proteins to increase intracellular calcium, required for virus replication and cell death induction. In Proceedings of the National Academy of Sciences of the United States of America; National Academy of Sciences: Washington, DC, USA, 2012; Volume 109, pp. 18471–18476. [CrossRef]
15. Casciano, J.C.; Duchemin, N.J.; Lamontagne, R.J.; Steel, L.F.; Bouchard, M.J. Hepatitis B virus modulates store-operated calcium entry to enhance viral replication in primary hepatocytes. *PLoS ONE* **2017**, *12*, e0168328. [CrossRef] [PubMed]
16. Berridge, M.J.; Bootman, M.D.; Roderick, H.L. Calcium signalling: Dynamics, homeostasis and remodelling. *Nat. Rev. Mol. Cell Biol.* **2003**, *4*, 517–529. [CrossRef]
17. Bergmeier, W.; Weidinger, C.; Zee, I.; Feske, S. Emerging roles of store-operated Ca2+entry through STIM and ORAI proteins in immunity, hemostasis and cancer. *Channels* **2013**, *7*, 379–391. [CrossRef]
18. Shaw, P.J.; Weidinger, C.; Vaeth, M.; Lüthy, K.; Kaech, S.M.; Feske, S. CD4+ and CD8+ T cell–dependent antiviral immunity requires STIM1 and STIM2. *J. Clin. Investig.* **2014**, *124*, 4549–4563. [CrossRef]
19. Chen, C.-J.; Yang, H.; Su, J.; Jen, C.-L.; You, S.-L.; Lu, S.-N.; Huang, G.-T.; Iloeje, U.H.; for the REVEAL-HBV Study Group. Risk of Hepatocellular Carcinoma Across a Biological Gradient of Serum Hepatitis B Virus DNA Level. *JAMA* **2006**, *295*, 65–73. [CrossRef]
20. Chan, H.L.-Y.; Tsang, S.W.-C.; Liew, C.-T.; Tse, C.-H.; Wong, M.-L.; Ching, J.Y.-L.; Leung, N.W.-Y.; Tam, J.S.-L.; Sung, J.J. Viral genotype and hepatitis B virus DNA levels are correlated with histological liver damage in HBeAg-negative chronic hepatitis B virus infection. *Am. J. Gastroenterol.* **2002**, *97*, 406–412. [CrossRef]
21. Yang, B.; Bouchard, M.J. The Hepatitis B Virus X Protein Elevates Cytosolic Calcium Signals by Modulating Mitochondrial Calcium Uptake. *J. Virol.* **2011**, *86*, 313–327. [CrossRef]
22. Yao, J.-H.; Liu, Z.-J.; Yi, J.-H.; Wang, J.; Liu, Y.-N. Hepatitis B Virus X Protein Upregulates Intracellular Calcium Signaling by Binding C-terminal of Orail Protein. *Curr. Med Sci.* **2018**, *38*, 26–34. [CrossRef]
23. Chang, W.-C.; Lee, C.-H.; Hirota, T.; Wang, L.-F.; Doi, S.; Miyatake, A.; Enomoto, T.; Tomita, K.; Sakashita, M.; Yamada, T.; et al. ORAI1 Genetic Polymorphisms Associated with the Susceptibility of Atopic Dermatitis in Japanese and Taiwanese Populations. *PLoS ONE* **2012**, *7*, e29387. [CrossRef]
24. Chou, Y.-H.; Juo, S.-H.H.; Chiu, Y.-C.; Liu, M.-E.; Chen, W.-C.; Chang, C.-C.; Chang, W.-P.; Chang, J.-G.; Chang, W.-C. A Polymorphism of the ORAI1 Gene is Associated with the Risk and Recurrence of Calcium Nephrolithiasis. *J. Urol.* **2011**, *185*, 1742–1746. [CrossRef]
25. Wei, J.C.-C.; Yen, J.-H.; Juo, S.-H.H.; Chen, W.-C.; Wang, Y.-S.; Chiu, Y.-C.; Hsieh, T.-J.; Guo, Y.-C.; Huang, C.-H.; Wong, R.-H.; et al. Association of ORAI1 Haplotypes with the Risk of HLA-B27 Positive Ankylosing Spondylitis. *PLoS ONE* **2011**, *6*, e20426. [CrossRef]

26. Thiha, K.; Mashimo, Y.; Suzuki, H.; Hamada, H.; Hata, A.; Hara, T.; Tanaka, T.; Ito, K.; Onouchi, Y.; Japan Kawasaki Disease Genome Consortium. Investigation of novel variations of ORAI1 gene and their association with Kawasaki disease. *J. Human Genet.* **2019**, *64*, 511–519. [CrossRef]
27. Wang, J.-Y.; Sun, J.; Huang, M.-Y.; Wang, Y.-S.; Hou, M.-F.; Sun, Y.; He, H.; Krishna, N.; Chiu, S.-J.; Lin, S.; et al. STIM1 overexpression promotes colorectal cancer progression, cell motility and COX-2 expression. *Oncogene* **2015**, *34*, 4358–4367. [CrossRef]
28. Zhang, Z.; Liu, X.; Feng, B.; Liu, N.; Wu, Q.; Han, Y.; Nie, Y.; Wu, K.; Shi, Y.; Fan, D. STIM1, a direct target of microRNA-185, promotes tumor metastasis and is associated with poor prognosis in colorectal cancer. *Oncogene* **2014**, *34*, 4808–4820. [CrossRef] [PubMed]
29. Chen, Y.F.; Chiu, W.T.; Chen, Y.T.; Lin, P.Y.; Huang, H.J.; Chou, C.Y.; Chang, H.C.; Tang, M.J.; Shen, M.R. Calcium store sensor stromal-interaction molecule 1-dependent signaling plays an important role in cervical cancer growth, migration, and angiogenesis. In Proceedings of the National Academy of Sciences of the United States of America; National Academy of Sciences: Washington, DC, USA, 2011; Volume 108, pp. 15225–15230. [CrossRef]
30. Sun, J.; Lu, F.; He, H.; Shen, J.; Messina, J.L.; Mathew, R.; Wang, D.; Sarnaik, A.A.; Chang, W.-C.; Kim, M.; et al. STIM1- and Orai1-mediated Ca2+ oscillation orchestrates invadopodium formation and melanoma invasion. *J. Cell Biol.* **2014**, *207*, 535–548. [CrossRef] [PubMed]
31. Huang, W.-C.; Chai, C.-Y.; Chen, W.-C.; Hou, M.-F.; Wang, Y.-S.; Chiu, Y.-C.; Lu, S.-R.; Chang, W.-C.; Juo, S.-H.H.; Wang, J.-Y.; et al. Histamine regulates cyclooxygenase 2 gene activation through Orai1-mediated NFκB activation in lung cancer cells. *Cell Calcium* **2011**, *50*, 27–35. [CrossRef]
32. Yang, S.; Zhang, J.J.; Huang, X.-Y. Orai1 and STIM1 Are Critical for Breast Tumor Cell Migration and Metastasis. *Cancer Cell* **2009**, *15*, 124–134. [CrossRef] [PubMed]
33. Feske, S. ORAI1 and STIM1 deficiency in human and mice: Roles of store-operated Ca2+entry in the immune system and beyond. *Immunol. Rev.* **2009**, *231*, 189–209. [CrossRef]
34. Feske, S. Immunodeficiency due to defects in store-operated calcium entry. *Ann. N. Y. Acad. Sci.* **2011**, *1238*, 74–90. [CrossRef]
35. Koike, K.; Tsutsumi, T.; Fujie, H.; Shintani, Y.; Moriya, K. Molecular Mechanism of Viral Hepatocarcinogenesis. *Oncology* **2002**, *62*, 29–37. [CrossRef]
36. Bootman, M.D.; Collins, T.J.; Peppiatt, C.M.; Prothero, L.S.; Mac Kenzie, L.; De Smet, P.; Travers, M.; Tovey, S.C.; Seo, J.T.; Berridge, M.J.; et al. Calcium signalling—An overview. *Semin. Cell Dev. Biol.* **2001**, *12*, 3–10. [CrossRef]