PROMOTER METHYLATION REGULATES MAGE-A3 EXPRESSION IN HEPATOCELLULAR CARCINOMA

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ABSTRACT
Hepatocellular carcinoma (HCC) represents one of the most common causes of cancer-related death worldwide, with few available treatment options. Cancer-testis antigens (CTAs), notably from the *MAGE* gene family, have opened new possibilities for improving immunotherapy in HCC due to their tumor-specific expression and high immunogenicity. However, the mechanisms regulating *MAGE* gene expression are largely unknown. This study explored mechanisms controlling the expression of *MAGE-A3* and *MAGE-C2*. We utilized HCC organoid cultures derived from patient HCC tissues known to represent cancer epigenetic status more accurately than cell lines to study the *MAGE* gene family expression patterns and promoter methylation patterns. Our findings reveal that *MAGE-A3* expression is correlated with hypomethylation of its promoter region, whereas *MAGE-C2* expression does not show such a correlation, suggesting other epigenetic mechanisms may regulate it. These findings suggest possibilities for targeting DNA methylation to control *MAGE-A3* expression in HCC. This study supports the exploration of CTAs in cancer immunotherapy and underscores the complexity of *MAGE* gene regulation in HCC.
Keywords: Hepatocellular Carcinoma, Cancer-Testis Antigen, Organoid and DNA Methylation

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related illness and death, contributing to the second-highest number of cancer fatalities worldwide (Sim & Knox, 2018). Current treatments for HCC, such as surgery, chemotherapy, and radiofrequency ablation, often fail to eliminate residual cancer cells (Raza & Sood, 2014). Active research into immunotherapeutic approaches for HCC, including CAR T cells and cancer vaccines, is underway (Fasano et al., 2021).

Cancer testis antigens (CTAs) are tumor-associated antigens encoded by non-mutated genes that are overexpressed in tumor cells but are rarely found in normal adult tissues. Their high tumor specificity and strong immunogenicity make them ideal targets for immunotherapy (Wagner et al., 2018). The first successful cloning of a human tumor antigen, melanoma antigen 1 (*MAGE1*), which triggered an autologous cytotoxic T lymphocyte (CTL) response in a melanoma patient, was reported by Boon et al. in the early 1990s (Duperret et al., 2018; Traversari et al., 1992). Studies have identified CTAs like *MAGE-A3* and *MAGE-C2* as potential targets for HCC immunotherapy. *MAGE-A3* is expressed in approximately 68% of HCC cases, while *MAGE-C2* is expressed in 74.6% of cases (Gu et al., 2019; Tahara et al., 1999; Wang et al., 2015). Survival analysis suggests that the expression of these antigens is an independent prognostic factor for HCC patients (Qiu, et al., 2006; Wang et al., 2015). However, the mechanisms controlling *MAGE* gene expression, including their re-expression in cancer, remain largely unexplored.

A 1999 melanoma cell line study identified DNA methylation as a mechanism regulating *MAGE* gene expression. However, demethylation agents like 5-aza-2-deoxycytidine (5DC) were not always effective in inducing *MAGE* gene expression in specific cell types (De Smet et al., 1999; Weon & Potts, 2015). Research on the epigenetic regulation of *MAGE-A3* and *MAGE-C2* in HCC has been limited to studies on cancer cell lines, which often change during their passage, resulting in findings that may not represent the original tumor (Qiu, et al., 2006). Organoids have become promising models for cancer research as they mimic patient-specific tissue features and functions in the lab. Organoid cultures offer several advantages over traditional two-dimensional cell cultures and animal models, including ease of manipulation and detailed study, making them widely used in drug discovery, diagnostics, and cellular research (Sato et al., 2011). Preliminary research has shown that the responses of cancer organoids are similar to those observed in patients.

This study explores the mechanisms regulating CTA expression using HCC organoids derived from liver cancer tissue samples of nine HCC patients. We analyze the transcriptome through RNA sequencing to identify expression patterns associated with CTA genes and HCC. These findings are validated through qPCR, and we investigate the regulatory mechanisms of these gene expressions. Our results provide insights into the mechanisms of epigenetic regulation of these genes in HCC and highlight their potential as therapeutic targets.

RESEARCH METHODOLOGY

Cell culture and RNA-seq

For the cell culture of HCC organoid DNA samples, we collected 9 HCC samples sourced from adult liver tissue of HCC patients. The microenvironment was mimicked using extracellular matrix (ECM) or scaffold to support cell growth. Organoid growth and differentiation were regulated by specific growth factors and signaling pathways. The culture media were supplemented with growth factors, cytokines, and small molecules to promote organoid formation, expansion, and differentiation.

The process began with the isolation or generation of stem cells or progenitor cells from the target liver tissue. These cells were embedded in the appropriate ECM or scaffold and cultured under conditions conducive to organoid growth and development. Organoids were typically

cultured in specialized incubators under controlled conditions, including temperature, humidity, and 5% CO2.

For cell culture for HCC cell lines, we use HepG2 and HEK293.The cell culture was maintained by DMEM high glucose medium (Cytiva) supplemented with 10% Fetal Bovine Serum (FBS), 1% Antibiotic-Antimycotic, and 1% GlutaMAX™ at 37°C. Then extract DNA by DNeasy® Blood & Tissue Kit (Qiagen)

After sufficient growth, the organoids were harvested, and RNA was extracted using the Qiagen RNA extraction kit. The extracted RNA was then stored at -80°C for future use. The RNA samples were subsequently sent for RNA sequencing analysis to assess gene expression profiles.

qPCR

To synthesize cDNA from RNA, we first prepared a master mix containing 100-1000 ng of RNA template, reverse transcriptase buffer, dNTPs (deoxynucleotide triphosphates), oligo(dT) primer, reverse transcriptase enzyme, RNase inhibitor (Riboblock), and nuclease-free water. This mixture was incubated in a thermal cycler under the following conditions: 25°C for 10 minutes, 42-50°C for 30-60 minutes, and 70°C for 10 minutes.

For qPCR analysis, we prepared a reaction mix by combining the cDNA template, SYBR Green Master Mix, forward and reverse primers specific for *MAGE-A3*, and *MAGE -C2*, and nuclease-free water. Primers were designed to ensure specificity and efficiency for qPCR amplification. The reaction mix was aliquoted into a 96-well qPCR plate, with triplicate wells for each sample and control. The thermal cycling program used was as follows: initial denaturation at 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. Data analysis was performed using the 2^-ΔΔCt method to determine relative gene expression levels. This method allows for the effective synthesis of cDNA from RNA and the subsequent qPCR analysis of *MAGE-A3*, and *MAGE-C2* expression in the samples.

Bisulfite PCR

For bisulfite conversion of DNA, we utilized the MethylEdge® Bisulfite Conversion System from Promega. We began by adding 20 µl of DNA samples to 130 µl of Bisulfite ME Conversion Reagent, followed by a quick centrifugation to collect the mixture at the tube's bottom. The samples were then incubated in a thermocycler under the following conditions: 8 seconds at 98 °C, 60 minutes at 54 °C, and then held at 4 °C. Post-incubation, we added 600 µl of ME Binding Buffer to the bisulfite-treated samples in ME Spin Columns, inverted the tubes, and centrifuged at maximum speed for 30 seconds. We washed the samples by adding 100 µl of 1X ME Wash Buffer and spinning for 30 seconds. We then added 200 µl of ME Desulphonation Buffer and incubated for 15 minutes at room temperature, followed by a wash with 200 µl of ME Wash Buffer, repeated twice. Finally, we eluted the DNA with 20 µl of ME Elution Buffer, centrifuged at maximum speed for 30 seconds.

We use Platinum Taq for the amplification of DNA (Invitrogen™). Perform PCR amplification with 25–35 cycles of 30 seconds at 94°C for denaturing, 30 seconds at 55°C for annealing, and 1 minute per kb at 72°C for extension. After cycling, keep the reaction at 4°C. Store the samples at -20°C until use and analyze the products using agarose gel electrophoresis. Then sent to sequencing process by plasmid

RESEARCH RESULTS

Heterogeneity of HCC organoids in *MAGE-A3* and *MAGE-C2* expression: classification into high and low expression groups

From the results of our HCC organoid culture and derivation process, we observed significant heterogeneity among the organoids sourced from tissues with different pathological backgrounds table1. The HCC organoids we developed exhibited cellular structures and arrangements closely resembling those of the original tissue Figure1. Additionally, these

organoids expressed alpha-fetoprotein (AFP), a specific protein marker commonly associated with HCC.

Table 1 HCC organoid samples with different and patients, backgrounds

No.	Gender	Age	HCC differentiated
N1	Male	70	Moderately
N5	Male	63	Poorly
N8	Female	60	Poorly
N9	Male	69	Moderately
N13	Male	61	Moderately
N17	Male	65	Moderately
N19	Male	62	Moderately
N22	Male	37	Poorly
N34	Male	60	Moderately

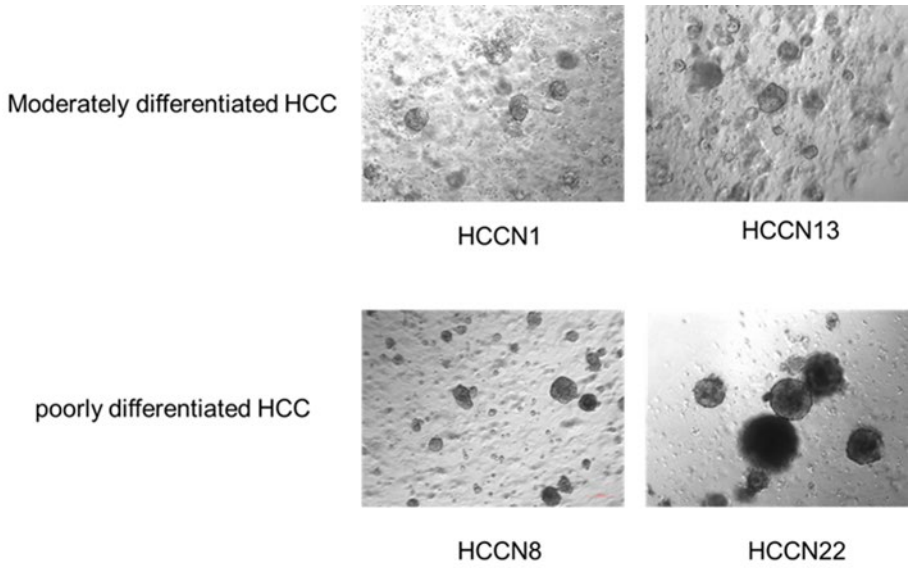

Figure 1 Phase contrast microscopy images of HCC organoids cultured from tissues with different pathological characteristics.

These organoids are further analysis in expression level, we observed distinct expression profiles of these CTA genes across the categorized groups. Figure 2 illustrates that HCC Groups 0 and 1 displayed relatively low or no expression of these genes. Of particular interest was the observation in HCC Group 2. Within this group, samples N19, N22, N5, and N8 exhibited significantly elevated expression levels of *MAGE-A3*, and *MAGE-C2*. Specifically, for *MAGE-A3*, N22 exhibited the highest peak of expression, followed by N19, N8, and N5, with N5 showing the smallest peak of expression. In the case of *MAGE-C2*, N5 showed the highest peak of expression, followed by N19, with N22 and N8 exhibiting similar levels.
This notable expression pattern highlights the potential relevance of these samples, particularly those in HCC Group 2, for further study in the context of HCC immunotherapy. The elevated expression levels of *MAGE-A3*, and *MAGE-C2* in these samples underscore their potential as therapeutic targets in HCC.

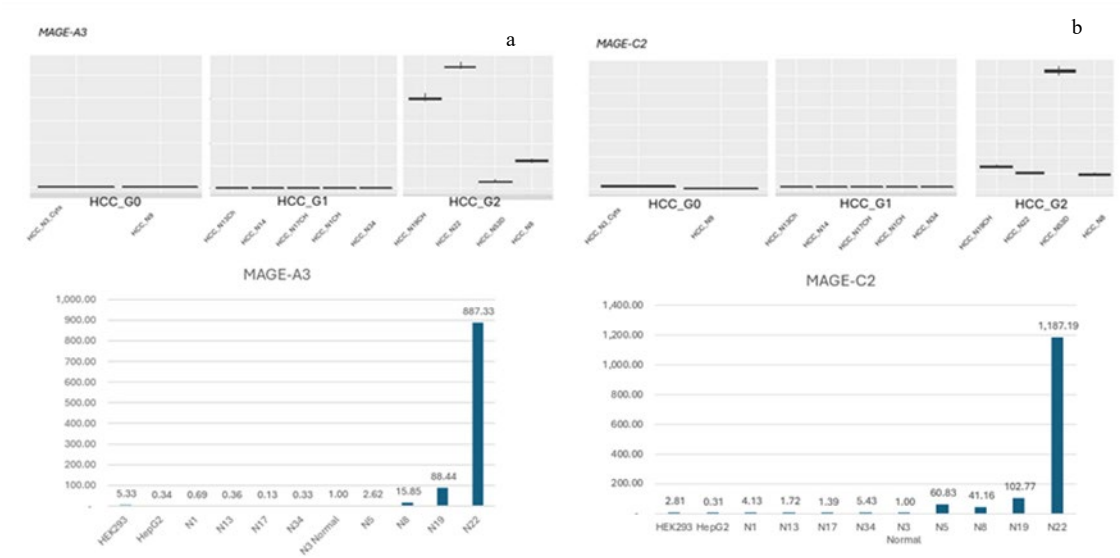

Figure 2 a. The expression in *MAGE-A3* gene in HCC samples by RNA sequencing and qPCR., b. The expression in *MAGE-C2* gene in HCC samples by RNA sequencing and qPCR.

Following RNA sequencing analysis, four samples emerged as potential candidates with significant expression patterns indicative of hepatocellular carcinoma (HCC). To validate these findings, we conducted qPCR assays to compare the expression levels of *MAGE-A3,* and *MAGE-C2* in these samples, alongside samples which low expression groups and sample N3 that represents a normal liver organoid.

The qPCR results, illustrated in Figure 2, revealed distinct expression profiles among the samples. Specifically, samples N1, N13, N3, N17 and N34 exhibited notably lower expression levels of *MAGE-A3*, and *MAGE-C2*. The 2^-ΔΔCt expression levels for *MAGE-A3* were 0.69, 0.36, 1.00, 0.13 and 0.33 respectively; and for *MAGE-C2* were 4.13, 1.72, 1.00, 1.39 and 5.43 respectively.

In contrast, samples N5, N8, N19, and N22, identified as potential HCC candidates based on RNA sequencing, displayed markedly higher expression levels of both genes. The 2^-ΔΔCt expression levels for *MAGE-A3* were 2.63, 15.85, 88.44, and 887.33, respectively; and for *MAGE-C2* were 60.83, 41.16, 102.77, and 1187.19, respectively.

The consistency between the qPCR and RNA sequencing data underscores the robustness of our findings. The significant upregulation of *MAGE-A3,* and *MAGE-C2* in HCC samples compared to the low expression and normal liver organoid samples reinforces the potential of these genes as target therapeutics for HCC. The concordance between the two techniques further validates the reliability of our results and highlights the identified samples as valuable targets for further investigation in the context of HCC immunotherapy.

Correlation between *MAGE-A3* expression and promoter region methylation

The DNA methylation profiles of *MAGE-A3* were analyzed using bisulfite conversion shown in figure3 and. Previous research suggested that the promoter region was the important part that contains CpG island for role of silencing genes and epigenetic regulation modified, so we investigate this DNA methylation pattern in promoter area of these genes.

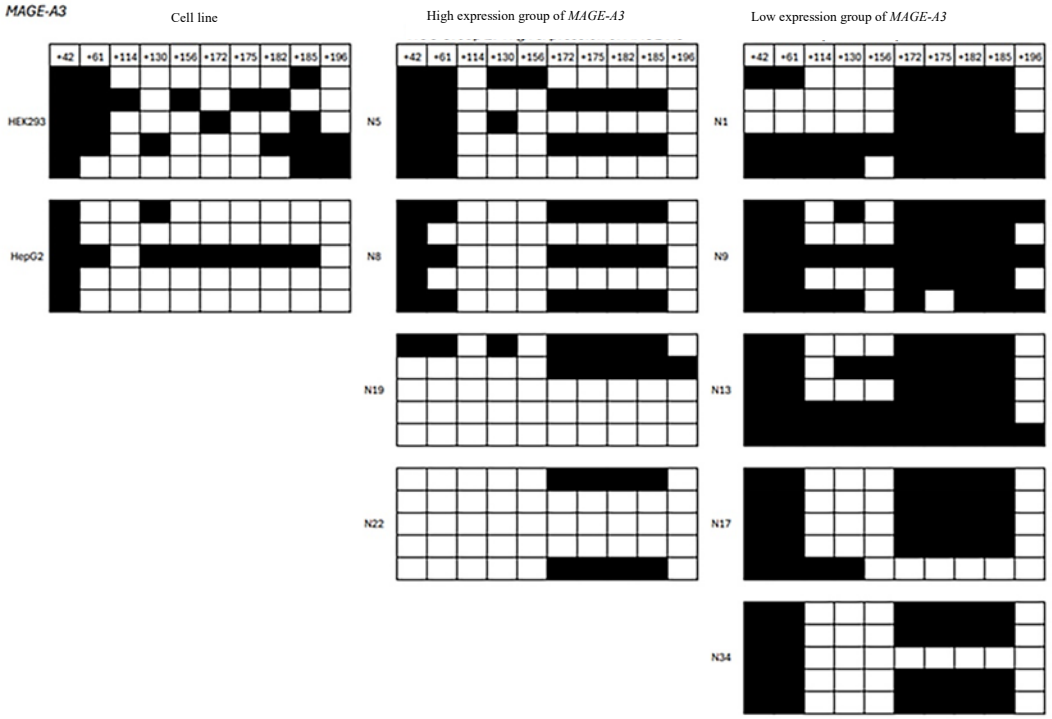

Figure 3 The DNA methylation pattern of Cell line (HEK293 and HepG2), HCC organoid with high expression of *MAGE-A3* (N5, N8, N19 and N22) and HCC organoid with low expression of *MAGE-A3* (N1, N9, N13, N17 and N34).

For *MAGE-A3*, methylation was assessed at several positions. In the high expression group (N5, N8, N19, N22) and the HCC cell line HepG2, positions +42 and +61 showed hypermethylation. Specifically, position +42 in sample N22 and position +61 in samples N19 and N22 were hypomethylated. Positions +172, +175, +182, and +185 were hypomethylated in samples N5, N8, and N22. In contrast, the low expression group (N1, N9, N13, N17 and N34) and the HEK293 cell line, which typically has low expression, exhibited hypermethylation at these positions. Notably, sample N13 from HCC low expression group showed vast hypermethylation also N1, N9, N17 and N34 showed hypermethylation in position+175, +182, and +185, correlating with its low expression profile observed in RNA sequencing.

These findings suggest that specific methylation patterns at these promoter positions of *MAGE-A3* may play a crucial role in regulating its expression in HCC. The hypomethylation in high-expression HCC samples and HepG2 cell line implies a potential epigenetic mechanism influencing gene activation in these contexts.

Lack of correlation between *MAGE-C2* expression and promoter region methylation

In contrast, the methylation analysis of *MAGE-C2* did not reveal significant differences between the high expression group and the low expression group, nor between the HCC cell lines (HepG2) and the HEK293 cell line that shows in figure 4. This lack of differential methylation suggests that the examined promoter regions of *MAGE-C2* may not play a significant role in regulating its expression in HCC.

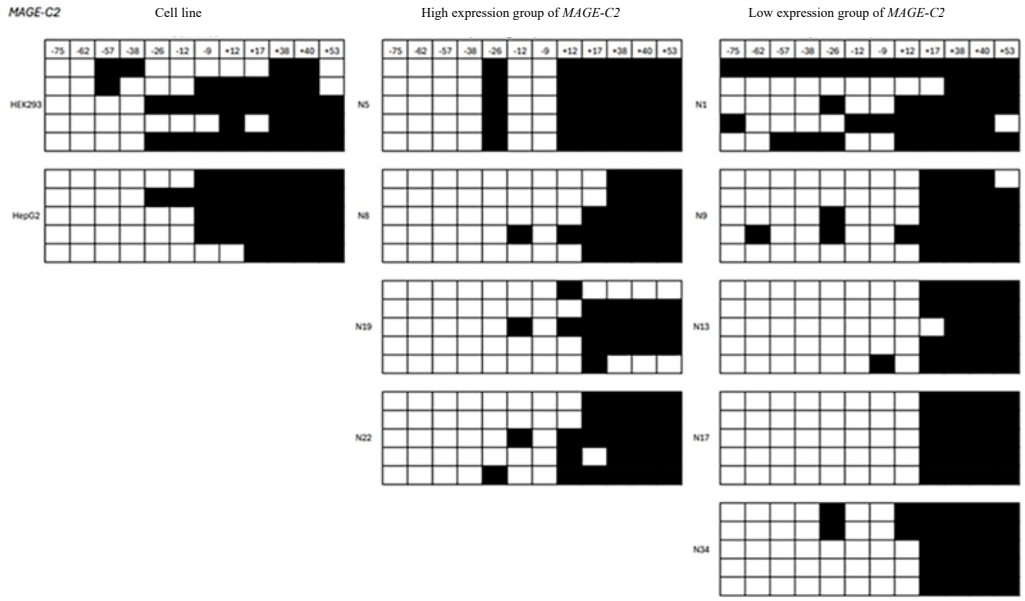

Figure 4 The DNA methylation pattern of Cell line (HEK293 and HepG2), HCC organoid with high expression of *MAGE-C2* (N5, N8, N19 and N22) and HCC organoid with low expression of *MAGE-C2* (N1, N9, N13, N17 and N34).

Overall, our results highlight the potential regulatory impact of DNA methylation on *MAGE-A3* expression in HCC, while indicating that *MAGE-C2* expression may be regulated by different mechanisms.

DISCUSSION & CONCLUSION

Our study examined the expression and regulation of *MAGE-A3* and *MAGE-C2* in hepatocellular carcinoma (HCC) organoids. We observed that *MAGE-A3* expression may be influenced by promoter region methylation, while *MAGE-C2* expression seems independent of this methylation status. These results indicate that DNA methylation is a critical regulatory factor for *MAGE-A3* in HCC but not for *MAGE-C2*. The methylation-dependent expression of *MAGE-A3* aligns with previous findings in melanoma, suggesting that this regulatory mechanism also applies to HCC. On the other hand, the regulation of *MAGE-C2* by alternative mechanisms underscores the complexity of gene regulation in cancer.

Our findings are consistent with those of De Smet et al. (1999) and Weon & Potts (2015), who demonstrated the role of DNA methylation in gene regulation in melanoma. However, our use of organoid models provides a more accurate reflection of in vivo conditions compared to cell lines, highlighting the value of organoids in cancer research. The methylation-dependent regulation of *MAGE-A3* suggests that targeting DNA methylation could be a promising strategy for HCC therapy. Given *MAGE-A3*'s strong immunogenicity and tumor-specific expression, it is an attractive candidate for immunotherapy. Further exploration of the alternative regulatory mechanisms governing *MAGE-C2* could lead to new therapeutic approaches.

This study is limited by the small sample size of nine HCC organoid cultures, which may not fully represent the heterogeneity of HCC. Additionally, organoids offer significant advantages over cell lines but cannot wholly replicate the tumor microenvironment. Future research should delve deeper into epigenetic factors regulating *MAGE-C2* expression using genome-wide methods. Investigating other epigenetic modifications, such as histone modifications, could further enhance our understanding of *MAGE* gene expression in HCC.

In conclusion, our study highlights the distinct regulatory mechanisms of *MAGE-A3* and *MAGE-C2* in HCC, emphasizing *MAGE-A3*'s potential as a therapeutic target. These findings contribute to a deeper understanding of CT antigen regulation in HCC and reinforce the use of organoids as a valuable model in cancer research.

REFERENCES

De Smet, C., Lurquin, C., Lethé, B., Martelange, V., & Boon, T. (1999). DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter. *Molecular and Cellular Biology, 19*(11), 7327-7335.

Duperret, E. K., Liu, S., Paik, M., Trautz, A., Stoltz, R., Liu, X., . . . Weiner, D. B. (2018). A Designer Cross-reactive DNA Immunotherapeutic Vaccine that Targets Multiple MAGE-A Family Members Simultaneously for Cancer Therapy. *Clinical Cancer Research, 24*(23), 6015-6027.

Fasano, R., Shadbad, M. A., Brunetti, O., Argentiero, A., Calabrese, A., Nardulli, P., . . . Silvestris, N. (2021). Immunotherapy for Hepatocellular Carcinoma: New Prospects for the Cancer Therapy. *Life (Basel), 11*(12).

Gu, X., Mao, Y., Shi, C., Ye, W., Hou, N., Xu, L., . . . Zhao, W. (2019). MAGEC2 Correlates With Unfavorable Prognosis and Promotes Tumor Development In HCC Via Epithelial-Mesenchymal Transition. *Onco Targets and Therapy, 12*, 7843-7855.

Qiu, G., Fang, J., & He, Y. (2006). 5′ CpG island methylation analysis identifies the MAGE-A1 and MAGE-A3 genes as potential markers of HCC. *Clinical Biochemistry, 39*(3), 259-266.

Raza, A., & Sood, G. K. (2014). Hepatocellular carcinoma review: current treatment, and evidence-based medicine. *World Journal of Gastroenterology, 20*(15), 4115-4127.

Sato, T., Stange, D. E., Ferrante, M., Vries, R. G., Van Es, J. H., Van den Brink, S., . . . Clevers, H. (2011). Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology, 141*(5), 1762-1772.

Sim, H. W., & Knox, J. (2018). Hepatocellular carcinoma in the era of immunotherapy. *Current Problems in Cancer, 42*(1), 40-48.

Tahara, K., Mori, M., Sadanaga, N., Sakamoto, Y., Kitano, S., & Makuuchi, M. (1999). Expression of the MAGE gene family in human hepatocellular carcinoma. *Cancer, 85*(6), 1234-1240.

Traversari, C., van der Bruggen, P., Luescher, I. F., Lurquin, C., Chomez, P., Van Pel, A., ... & Boon, T. (1992). A nonapeptide encoded by human gene MAGE-1 is recognized on HLA-A1 by cytolytic T lymphocytes directed against tumor antigen MZ2-E. *The Journal of Experimental Medicine, 176*(5), 1453-1457.

Wagner, S., Mullins, C. S., & Linnebacher, M. (2018). Colorectal cancer vaccines: Tumor-associated antigens vs neoantigens. *World Journal of Gastroenterology, 24*(48), 5418-5432.

Wang, M., Li, J., Wang, L., Chen, X., Zhang, Z., Yue, D., ... & Zhang, Y. (2015). Combined cancer testis antigens enhanced prediction accuracy for prognosis of patients with hepatocellular carcinoma. *International Journal of Clinical and Experimental Pathology, 8*(4), 3513-3528.

Weon, J. L., & Potts, P. R. (2015). The MAGE protein family and cancer. *Current Opinion in Cell Biology, 37*, 1-8.

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