Expression Levels of miR-590-3p in Hepatocellular Carcinoma and Osteosarcoma Cell Lines and its Downstream Target Genes

Mennatallah Mohamed Bassem Mahmoud Elfar

The American University in Cairo

Follow this and additional works at: https://fount.aucegypt.edu/etds

Recommended Citation

APA Citation

MLA Citation

This Master's Thesis is brought to you for free and open access by the Student Research at AUC Knowledge Fountain. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of AUC Knowledge Fountain. For more information, please contact mark.muehlhaeusler@aucegypt.edu.
School of Sciences and Engineering

Expression Levels of miR-590-3p in Hepatocellular Carcinoma and Osteosarcoma Cell Lines and its Downstream Target Genes

A Thesis Submitted to Biotechnology Master’s Program
In partial fulfillment of the requirements for the degree of Master of Science

By:
Mennatallah Elfar

Under the supervision of
Dr. Asma Amleh

September/2018
Expression Levels of miR-590-3p in Hepatocellular Carcinoma and Osteosarcoma Cell Lines and its Downstream Target Genes

A Thesis Submitted by

Mennatallah Elfar

To the Biotechnology Graduate Program

September/ 2018

In partial fulfillment of the requirements for

The degree of Master of Science

Has been approved by

Thesis Committee Supervisor/Chair

Affiliation

Thesis Committee Reader/Examiner

Affiliation

Thesis Committee Reader/Examiner

Affiliation

Thesis Committee Reader/External Examiner

Affiliation

Dept. Chair/Director Date Dean Date
Dedication

To my Family, for all their unconditional love and support!

First, to my Father and Mother, Bassem and Hanan, everything I am is for you and because of you; you mean the world to me. Second, to my brother, Amged, and my sister, Hagar, you’ve always had my back; thank you for everything. Third, to my brother in law, M. Ekram, and my sister in law, Rana, your support means a lot to me. Finally, to the newest edition to our growing family, my nephew, Eyad, you give meaning to our lives.
Acknowledgments

I would like to thank everyone who contributed to this work. First and foremost, I would like to express my deepest gratitude to my thesis advisor, Dr. Asma Amleh, for all her never-ending effort, support, guidance and patience during my thesis work. I am very thankful to have been part of her team and to grow as a scientist in her lab.

I would also like to thank all AUC professors in the Biotechnology Program who taught me. I would like to thank Mr. Amged Ouf and Mr. Ahmed El Hossieny for their help and support. I would like to express my gratitude to Heba Shawer for her endless help and support.

My deepest appreciation goes to my lifelong friends, Rana, Wafaa, Nada Hany, Yara, Salma Khaled and Yasmine and my amazing teammates and my second family, Myret, Sheri, Youssef, Nada El Zahed, Rowan and Menna G. I would also like to thank Nancy, Noha, Diana, Nahla, Hana, Amal, Marwa, Salma El Shafei and Eric for all their support.

Finally, I would like to thank AUC for providing me with research funds and fellowship opportunities.
Expression Levels of miR-590-3p in Hepatocellular Carcinoma and Osteosarcoma Cell Lines and its Downstream Target Genes

Abstract

Small non-coding RNA (miRNA) sequences of around 18-25 nucleotides can regulate different cellular pathways by acting on tumor suppressors, oncogenes or both. They are mostly tissue-specific and can be up-regulated or down-regulated, depending on the cancer or the tissue in which they are found. hsa-miR-590-3p was found to be involved in several types of cancers. In this study, we used computational analysis to identify potential downstream target genes of hsa-miR-590-3p. We used five databases (TargetScan, miRanda-mirSVR, miRDB, miRTarBase and Diana Tools) to obtain a dataset of potential downstream target genes of hsa-miR-590-3p from each database. We obtained lists of hundreds of genes from these databases. We obtained a shorter list of common genes in all five databases from which we selected fourteen genes with the potential to affect cancer progression and to help further understand the disease pathogenesis, based on previous literature and further in silico analysis using Expression Atlas and The Human Protein Atlas. SOX2, N-cadherin, E-cadherin and FOXA2 were utilized as potential downstream target genes of hsa-miR-590-3p. SNU449 and HepG2, hepatocellular carcinoma cell lines, and U2OS, osteosarcoma cell line, were used to carry out various molecular techniques to further validate our in silico results. mRNA and protein expression levels of these genes were detected using RT-PCR and western blotting respectively. In an attempt to co-localize the hsa-miR-590-3p and its candidate downstream target gene, SOX2, we conducted a miRNA in situ hybridization combined with immunohistochemistry staining using anti-SOX2. The results show that there is an inverse correlation between hsa-miR-590-3p expression and SOX2 protein expression in SNU449 and U2OS. Examining the expression of hsa-miR-590-3p downstream target genes can enhance our understanding of the cancer pathogenesis and how it can be utilized as a therapeutic tool.
TABLE OF CONTENTS

Dedication .......................................................................................................................... III
Acknowledgments ............................................................................................................... IV
Abstract ............................................................................................................................. V
Table of Contents .............................................................................................................. VI
Glossary and Abbreviations ............................................................................................. IX
List of Tables ......................................................................................................................... XII
List of Figures ..................................................................................................................... XIII

Chapter 1. Introduction ....................................................................................................... 1
  1.1. Liver Cancer ................................................................................................................. 1
      1.1.1. Incidence and epidemiology ..................................................................................... 1
      1.1.2. Hepatocellular Carcinoma: Etiology and Staging .................................................... 1
      1.1.3. Cellular and Molecular HCC .................................................................................. 2
  1.2. Bone cancer .................................................................................................................. 3
      1.2.1. Incidence and epidemiology .................................................................................... 3
      1.2.2. Osteosarcoma: Etiology ....................................................................................... 3
  1.3. Small non-coding RNAs ............................................................................................... 4
      1.3.1. miRNAs: Biogenesis and Function ......................................................................... 4
      1.3.2. Oncogenic Vs. Tumor Suppressive miRNAs ............................................................ 5
      1.3.3. miRNAs and the Hallmarks of Cancer .................................................................... 6
      1.3.4. hsa-miR-590-3p .................................................................................................... 8
  1.4. Scope of the Study and Study Objectives ................................................................... 8

Chapter 2. Materials and Methods ..................................................................................... 9
  2.1. In silico Analysis .......................................................................................................... 9
  2.2. Cell Culture ............................................................................................................... 10
  2.3. Viable Cell Count ....................................................................................................... 10
  2.4. Total RNA Extraction, Quantification and Integrity Analysis .................................... 11
  2.5. cDNA Synthesis and Semi-Quantitative RT-PCR ....................................................... 11
  2.6. Total Protein Extraction and Quantification ............................................................... 13
  2.7. Western Blotting ....................................................................................................... 13
2.8. *In situ* Hybridization – Immunocytochemistry (ISH-ICC).................................14
2.9. Statistical Analysis........................................................................................................15

**Chapter 3. Results**........................................................................................................15

3.1. Bioinformatic Analysis to assess the levels of hsa-miR-590-3p in HCC and OS and to Predict Potential Downstream Target Genes of hsa-miR-590-3p..............15
   3.1.1. Assessing the levels of hsa-miR-590-3p in HCC and OS.............15
   3.1.2. Primary Screening of Potential Targets of hsa-miR-590-3p..........15
   3.1.3. Function Prediction of Potential Targets of hsa-miR-590-3p........19
   3.1.4. Potential Targets of hsa-miR-590-3p using The Human Protein Atlas..............................................................21

3.2. mRNA Expression of Potential Downstream Target Genes of hsa-miR-590-3p.................................................................23
   3.2.1. mRNA Expression of the Fourteen Potential Targets of hsa-miR-590-3p in HCC cell lines..............................................23
   3.2.2. mRNA Expression of CX3CL1 in U2OS...........................................23

3.3. Bioinformatics Analysis to validate Other Potential Downstream Target Genes of hsa-miR-590-3p...............................................................................27
   3.3.1. Alignment of Potential Targets of hsa-miR-590-3p using miRanda-mirSVR.................................................................27
   3.3.2. mRNA and Protein Expression of Potential Targets of hsa-miR-590-3p using The Human Protein Atlas and Expression Atlas.....29

3.4. mRNA Expression of E-cadherin and N-cadherin as Potential Targets of hsa-miR-590-3p using RT-PCR and mRNA expression of Vimentin as a Mesenchymal Marker..............................................................31

3.5. Protein Expression of Vimentin as a Mesenchymal Marker..................32

3.6. mRNA and Protein Expression of SOX2 as a Potential Target of hsa-miR-590-3p........................................................................33

3.7. mRNA Expression of FOXA2 as a Potential Target of hsa-miR-590-3p and its Downstream Target Gene VCAN..............................................35

3.8. Expression and Localization of hsa-miR-590-3p and its Potential Target, SOX2, in U2OS and SNU449 cells.................................................................36
Chapter 4. Discussion ................................................................................................................. 40

4.1. Bioinformatic Analysis to Predict Potential Downstream Target Genes of hsa-miR-590-3p .................................................................................................................. 41
4.2. Potential Targets of hsa-miR-590-3p .................................................................................. 41
4.3. Potential Targets of hsa-miR-590-3p and Cell-cell Adhesion .............................................. 43
4.4 Potential Targets of hsa-miR-590-3p and the FOXA2-VCAN Pathway ................................. 46
4.5. Potential Targets of hsa-miR-590-3p and Cell Stemness .................................................... 47
4.6 Expression and Localization of hsa-miR-590-3p and SOX2 .................................................. 48

Chapter 5. Conclusion and Future Recommendations ................................................................. 49

5.1. Conclusion ........................................................................................................................... 49
5.2. Future Recommendations .................................................................................................... 49

References ....................................................................................................................................... 50

Appendix A: Downstream Target Genes of hsa-miR-590-3p obtained from TargetScan
Appendix B: Downstream Target Genes of hsa-miR-590-3p obtained from miRDB
Appendix C: Downstream Target Genes of hsa-miR-590-3p obtained from miRTarBase
Appendix D: Downstream Target Genes of hsa-miR-590-3p obtained from Diana Tools
Appendix E: Pivot Table
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline Phosphatase</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BCIP/NBT</td>
<td>5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium</td>
</tr>
<tr>
<td>BRCA</td>
<td>BReast CAncer susceptibility gene</td>
</tr>
<tr>
<td>BRIP1</td>
<td>BRCA1-interacting protein 1</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic acid</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>CTCF</td>
<td>Corrected Total Cell Fluorescence</td>
</tr>
<tr>
<td>CX3CL1</td>
<td>Fractalkine or chemokine (C-X3-C motif) ligand 1</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DCLRE1A</td>
<td>DNA cross-link repair 1A protein</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DIG</td>
<td>Digoxigenin</td>
</tr>
<tr>
<td>DLG1</td>
<td>Discs large homolog 1</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>DYRK2</td>
<td>Dual specificity tyrosine-phosphorylation-regulated kinase 2</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Epithelial Cadherin</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-Mesenchymal Transition</td>
</tr>
<tr>
<td>EOC</td>
<td>Epithelial Ovarian Cancer</td>
</tr>
<tr>
<td>ERCC5</td>
<td>Excision repair cross-complementing protein</td>
</tr>
<tr>
<td>FAME</td>
<td>Functional Assignment of microRNAs via Enrichment</td>
</tr>
<tr>
<td>FANCF</td>
<td>Fanconi anemia group F protein</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FOXA2</td>
<td>Forkhead box protein A2</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular Carcinoma</td>
</tr>
</tbody>
</table>
HepG2  Early-Stage Well Differentiated Human Hepatocellular Carcinoma cell line
HIPK2  Homeodomain-interacting protein kinase 2
ICC    Immunocytochemistry
ISH    In Situ Hybridization
MG-63  Human Bone Osteosarcoma Heterogeneous cell line
miRNA  microRNA
MLH3   DNA mismatch repair protein
mRNA   Messenger RNA
MTIs   miRNA Target Interaction
N-cadherin  Neural Cadherin
NGS    Next Generation Sequencing
nM     Nano Molar
nm     Nano meter
NPHP1  Nephrocystin-1
OS     Osteosarcoma
P-value Probability value
PCR    Polymerase Chain Reaction
RAD21  Double-strand-break repair protein
RNA    Ribonucleic acid
RPMI 1640 Roswell Park Memorial Institute medium
rRNA   Ribosomal RNA
RT-PCR Reverse Transcriptase - Polymerase Chain Reaction
Saos-2 Human Bone Osteosarcoma Epithelial cell line
SD     Standard Deviation
SDS    Sodium Dodecyl Sulfate
PAGE   Polyacrylamide Gel Electrophoresis
SMC6   Structural maintenance of chromosomes protein 6
SNU449 Intermediate stage Human Hepatocellular Carcinoma cell line
SOX2   SRY (sex determining region Y)-box 2
TBS    Tris-buffered Saline
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBST</td>
<td>0.01% Tween-20 in Tris-buffered Saline</td>
</tr>
<tr>
<td>TMEM33</td>
<td>Transmembrane Protein 33</td>
</tr>
<tr>
<td>TPM</td>
<td>Tags per Million</td>
</tr>
<tr>
<td>U2OS</td>
<td>Human Bone Osteosarcoma Epithelial cell line</td>
</tr>
<tr>
<td>UVRAG</td>
<td>UV radiation resistance-associated gene</td>
</tr>
<tr>
<td>VCAN</td>
<td>Versican</td>
</tr>
<tr>
<td>VIM</td>
<td>Vimentin</td>
</tr>
<tr>
<td>WB</td>
<td>Western Blotting</td>
</tr>
<tr>
<td>µL</td>
<td>Microliter</td>
</tr>
</tbody>
</table>
List of Tables

Table 1. Caner Staging using TNM system.................................................................2
Table 2. Examples of Tumor-Suppressive and Oncogenic miRNAs in Various Cancers.........................................................................................................................6
Table 3. Primers used in Semi-quantitative RT-PCR..............................................12
Table 4. Some of the Top Potential Downstream Target Genes of hsa-miR-590-3p obtained from TargetScan based on Total Context++ Score.........................................................17
Table 5. The Top Ranked Potential Downstream Target Genes of hsa-miR-590-3p obtained from miRDB based on Target Score......................................................................................17
Table 6. The Top Ranked Potential Downstream Target Genes of hsa-miR-590-3p obtained from miRTarBase based on validation method..............................................18
Table 7. The Top Ranked Potential Downstream Target Genes of hsa-miR-590-3p obtained from Diana Tools Based on miTG Score.........................................................18
Table 8. The Chosen Functions of the Potential Downstream Target Genes of hsa-miR-590-3p obtained from FAME Software ..................................................................................20
List of Figures

Figure 1. Illustration of miRNAs’ Biogenesis and Function..................................................5
Figure 2. The Six Main Hallmarks of Cancer............................................................................7
Figure 3. Primary Screening.....................................................................................................19
Figure 4. Function Prediction..................................................................................................21
Figure 5. Protein Expression of Potential Targets of hsa-miR-590-3p using The Human Protein Atlas in Normal versus Cancerous Liver Tissue ..........................................................22
Figure 6. Categorization of the Fourteen Genes Based on the Function they Share........22
Figure 7. mRNA Expression of Potential Downstream Target Genes of miR-590-3p in HepG2 and SNU449 using RT-PCR...........................................................................................................24
Figure 8. mRNA Expression of CX3CL1 in U2OS, HepG2 and SNU449 using RT-PCR........................................................................................................................................27
Figure 9. Alignment of the mRNA of Potential Targets of hsa-miR-590-3p against hsa-miR-590-3p using miRanda-miRsvR.........................................................................................................................28
Figure 10. mRNA Expression of Potential Targets of hsa-miR-590-3p in U2OS and HepG2 using The Human Protein Atlas..........................................................................................................................29
Figure 11. Protein Expression of Potential Targets of hsa-miR-590-3p in OS and HCC cell lines using Expression Atlas............................................................................................................................30
Figure 12. mRNA Expression of E-cadherin, N-cadherin and Vimentin in U2OS, HepG2 and SNU449 using RT-PCR...............................................................................................................................31
Figure 13. Protein Expression of Vimentin in U2OS, HepG2 and SNU449 using Western Blotting................................................................................................................................................33
Figure 14. mRNA and Protein Expression of SOX2 in U2OS, HepG2 and SNU449 using RT-PCR and Western Blotting Respectively..................................................................................................................34
Figure 15. mRNA Expression of FOXA2 and VCAN in U2OS, HepG2 and SNU449 using RT-PCR....................................................................................................................................................35
Figure 16. Expression and Localization of hsa-miR-590-3p and SOX2 in U2OS cells...37
Figure 17. Expression and Localization of hsa-miR-590-3p and SOX2 in SNU449 cells........................................................................................................................................38
Figure 18. Graphical Representation of the Expression of hsa-miR-590-3p and SOX2 in U2OS and SNU449 cells...

Figure 19. Illustration of Epithelial-Mesenchymal Transition...
1. Introduction

1.1. Liver Cancer

1.1.1. Incidence and epidemiology

Cancer is considered a worldwide epidemic. Globally, liver cancer is the second leading cause of cancer deaths (W. Ma et al., 2018). According to the American Cancer Society, since 1980, liver cancer incidence has more than tripled. Each year, more than 700,000 new cases of liver cancer are diagnosed. According to GLOBOCAN, the less developed countries account for 83% of liver cancer cases. Liver cancer is more prevalent in men than women (“American Cancer Society,” 2018). Hepatocellular carcinoma is the most common primary tumor of liver cancer cases (Ghouri, Mian, & Rowe, 2017).

1.1.2. Hepatocellular Carcinoma: Etiology and Staging

HCC is also known as malignant hepatoma. It is usually secondary to liver cirrhosis due to viral hepatitis infection (HBV and HCV), alcoholism or exposure to high levels of aflatoxin-b1 (AFB). HCC is heterogeneous, meaning many aspects must be taken in consideration when attempting to diagnose or treat it. For example, the tumor size, the number of liver vessels involved, the presence or absence of daughter nodules, the presence or absence of extra-hepatic metastasis and the vascularity of the tumor (Marrero, Kudo, & Bronowicki, 2010). All these factors are used to identify the stage of the disease. The TNM system is the most widely used system today (Table 1). TNM refers to the tumor size, the number of lymph nodules and whether the cancer metastasized or not. The numbers 0-4 refer to severity; with 4 being the most severe case, while X means the information is not available hence, it cannot be assessed (“American Cancer Society,” 2018).
Table 1. Caner Staging using TNM System.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Stage Grouping</th>
<th>Stage Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>T1, N0, M0</td>
<td>A single tumor that has not grown into any blood vessels and no spreading of the cancer to lymph nodes or distant sites</td>
</tr>
<tr>
<td>II</td>
<td>T2, N0, M0</td>
<td>A single tumor that has grown into a blood vessel or more than one tumor that has not grown into any blood vessels and no spreading of the cancer to lymph nodes or distant sites</td>
</tr>
<tr>
<td>IIIA</td>
<td>T3a, N0, M0</td>
<td>More than one tumor and no spreading of the cancer to lymph nodes or distant sites</td>
</tr>
<tr>
<td>IIIB</td>
<td>T3b, N0, M0</td>
<td>At least one tumor has grown into a branch of a major vein of the liver and no spreading of the cancer to lymph nodes or distant sites</td>
</tr>
<tr>
<td>IIIC</td>
<td>T4, N0, M0</td>
<td>The tumor has grown into a nearby organ and no spreading of the cancer to lymph nodes or distant sites</td>
</tr>
<tr>
<td>IVA</td>
<td>Any T, N1, M0</td>
<td>The tumor has grown into a blood vessel or a nearby organ and spread to lymph nodes but not distant sites</td>
</tr>
<tr>
<td>IVB</td>
<td>Any T, Any N, M1</td>
<td>The tumor has spread to distant sites regardless of the tumor size and whether or not lymph nodes are involved</td>
</tr>
</tbody>
</table>

Adapted from The American Cancer Society

1.1.3. Cellular and Molecular HCC

In HCC a number of factors contribute to facilitate the disease progression; starting from tumor initiation to metastasis. These factors include, the tumor’s microenvironment, hypoxia, inflammation and oxidative stress (Aravalli, Cressman, & Steer, 2013). Obesity, smoking and diabetes can also contribute to the development of HCC. In chronic inflammation and oxidative stress, cytokines and reactive oxygen
species respectively are released in the organ’s microenvironment resulting in genetic changes. The gradual accumulation of mutations changes the hepatocytes genetically resulting in gene expression alterations affecting various signaling pathways causing liver damage and eventually cancer development (Kumar, Zhao, & Wang, 2011).

1.2. Bone Cancer

1.2.1. Incidence and epidemiology

Bone cancer is not as prevalent as liver cancer. Bone tumors account for 3-5% of childhood cancers and less than 1% of adult cancers (Mirabello, Troisi, & Savage, 2009). Osteosarcoma (OS) is the most common bone cancer. Each year, around 800-900 new cases of OS are diagnosed in the United States only with more than half of the cases are children and adolescents (“American Cancer Society,” 2018).

1.2.2. Osteosarcoma: Etiology

Osteosarcoma is also known as osteogenic sarcoma. Several studies have been carried out attempting to determine the cause of OS. Unfortunately, the etiology of OS is still unknown, however there are some risk factors that are associated with OS. These risk factors include alkylating agents, ionizing radiation and some chromosomal abnormalities, for example, Paget’s disease, Li-Fraumeni cancer syndrome and hereditary retinoblastoma (Stanitski, 1997; Liang, Gao, Fu, Xu, & Qian, 2013).
1.3. Small non-coding RNAs

1.3.1. miRNAs: Biogenesis and Function

miRNAs are small non-coding RNA sequences of 18-25 nucleotides that can post-transcriptionally regulate different cellular pathways including cellular differentiation, growth, proliferation, metabolism, angiogenesis, regeneration, survival, apoptosis and tumorigenesis. Different miRNAs can act as tumor suppressors, oncogenes or both through the regulation of their downstream target genes. They are tissue-specific and can be up-regulated or down-regulated, depending on the cancer or the tissue in which they are found (Nana-Sinkam, 2011).

Most miRNA genes are located on intergenic regions (non-coding regions) in the nucleus and are expressed in the cytoplasm through the following process. In the nucleus, RNA polymerase II transcribes a long primary miRNA (pri-miRNA) with a hairpin-like structure, which is then converted to precursor miRNA (pre-miRNA), a 70-nucleotide stem loop, by RNase III endonuclease (RNASEN), known as Drosha in vertebrates. Exportin 5, a double-stranded RNA binding protein, transports the pre-miRNA to the cytoplasm, where Dicer cleaves it into 22-nucleotide double-stranded miRNAs. Then it is separated into two single-stranded miRNAs. The sense (passanger) strand is degraded while the anti-sense (guide) strand binds to RNA-induced silencing complex (RISC) as it is the strand with lower free energy. The interaction between 5’ seed sequence of the miRNA (position 2 to 8) and the 3’-untranslated region (3’-UTR) of the mRNA determines the effect of the miRNA, whether degradation of the mRNA or the inhibition of its translation. Many other proteins are involved in the RISC complex to degrade or silence mRNA targets, including helicases, mRNA decapping proteins, methytransferases, deadenylases and argonautes (Abba, Patil, & Allgayer, 2014). This process is further elaborated in figure 1.
Figure 1. Illustration of miRNAs’ Biogenesis and Function. In the nucleus, RNA polymerase II transcribes the pri-miRNA, which is then converted to pre-miRNA by Drosha. Exportin 5 transports the pre-miRNA to the cytoplasm, where Dicer cleaves it into 22-nucleotide double-stranded miRNAs. Then it is separated into two single-stranded miRNAs. The sense (passenger) strand is degraded while the anti-sense (guide) strand binds to RISC and inhibits the translation of the mRNA or degrades it. The figure was generated using Microsoft PowerPoint.

1.3.2. Oncogenic Vs. Tumor Suppressive miRNAs

According to miRBase, there are 1917 precursor and 2654 mature human miRNAs (Kozomara & Griffiths-Jones, 2014). Different miRNAs can act as tumor suppressors, oncogenes or both through the regulation of their downstream target genes. They are tissue-specific and can be up regulated or down-regulated, depending on the type of cancer or the tissue in which they are found. Various oncogenic miRNAs are overexpressed in different cancers while many tumor suppressive miRNAs are down-
regulated or lost in different cancers. Examples of oncogenic and tumor suppressive miRNAs in various cancers are listed in Table 2 (Gartel & Kandel, 2008).

Table 2. Examples of Tumor-Suppressive and Oncogenic miRNAs in Various Cancers.

<table>
<thead>
<tr>
<th>MiRNA</th>
<th>Alteration in cancers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor Suppressive</td>
<td></td>
</tr>
<tr>
<td>MiR-124a</td>
<td>Reduced by methylation in colon and lung cancers</td>
</tr>
<tr>
<td>Let-7</td>
<td>Reduced in lung and colon cancers</td>
</tr>
<tr>
<td>MiR-15a</td>
<td>Lost in CCL, pituitary adenoma</td>
</tr>
<tr>
<td>Oncogenic</td>
<td></td>
</tr>
<tr>
<td>MiR-21</td>
<td>Overexpressed in breast cancer, glioblastoma, CLL and cervical cancer</td>
</tr>
<tr>
<td>MiR-221</td>
<td>Overexpressed in thyroid cancer, glioblastoma, pancreatic cancer and prostate cancer</td>
</tr>
<tr>
<td>MiR-106a, miR-106 and miR-363</td>
<td>Overexpressed in T-cell leukemia, colon, pancreatic and prostate tumors</td>
</tr>
</tbody>
</table>

1.3.3. miRNAs and the Hallmarks of Cancer

Various miRNAs affect at least one of the six hallmarks of cancer: 1) sustaining growth signals, 2) insensitivity to growth suppressors, 3) enabling replicative immortality, 4) resisting apoptosis, 5) sustained angiogenesis and 6) invasion and metastasis (Figure 2) (s Hanahan and Weinberg, 2011). By targeting regulators of the cell cycle, miRNAs can independently promote or inhibit cell growth and proliferation by affecting the first three hallmarks of cancer (sustaining growth signals, insensitivity to growth suppressors and enabling replicative immortality). Examples of these miRNAs include let-7, miR-17, miR-221/222 and miR-21. MiRNAs can also target components of the apoptotic pathway thus affecting the fourth hallmark of cancer (resisting apoptosis). MiR-15a and miR-16-1 are examples of miRNAs that affect cell survival. Hypoxia, angiogenic factors and vascular endothelial growth factors (VEGF) affect the fifth hallmark of cancer (tumor
angiogenesis), therefore both tumor suppressive and oncogenic miRNAs control angiogenesis. The sixth hallmark of cancer (tissue invasion and metastasis) is controlled by several factors, including cellular proliferation and epithelial mesenchymal transition (EMT), which leads to cell motility, invasion and hematogenous spread. MiRNAs affect EMT and their expression has been used to differentiate between primary and metastatic tumors (Hanahan and Weinberg, 2011).

Figure 2. The Six Main Hallmarks of Cancer. Adapted from Hanahan and Weinberg, 2011.
1.3.4. hsa-miR-590-3p

Several studies tested hsa-miR-590-3p in relation to various cancers. miRNAs are tissue-specific, meaning that the same miRNA can be up-regulated in a specific tissue and down-regulated in another. This is the case with hsa-miR-590-3p. In glioblastoma multiforme (GBM), an aggressive brain cancer, hsa-miR-590-3p was significantly down-regulated in cancer tissue compared to normal tissues (Pang, Zheng, Zhao, Xiu, & Wang, 2015) while in epithelial ovarian cancer (EOC), hsa-miR-590-3p was significantly up-regulated in cancer tissue compared to that of the normal ovarian tissues (Salem et al., 2018). These differences in the expression suggest that hsa-miR-590-3p can regulate tumor suppressors and oncogenes as its downstream target genes depending on the cancer type.

Two studies reported the expression of hsa-miR-590-3p in HCC. In the first study, hsa-miR-590-3p was significantly down-regulated in cancer tissue compared to normal tissues (Ge & Gong, 2017) while in the second study, it was reported to be significantly up-regulated in three HCC cell lines (HepG2, Hep3B and Huh7) (Hongfei, Wenhong, Wentao, Chunyan, & Jing, 2013). There are reports of studies that assessed the expression levels of hsa-miR-590-3p in OS.

1.4. Scope of the Study and Study Objectives

miR-590-3p was reported to be expressed in HCC. However, its direct downstream target genes are not fully identified and understood. In this study, we employed various bioinformatics analysis to assess the levels of hsa-miR-590-3p in HCC and OS and to identify the potential downstream target genes of hsa-miR-590-3p and to predict their function in relation to both cancers. The main aim of the study is to assess the levels of hsa-miR-590-3p and the role of its downstream target genes in HCC and OS.
The specific objectives of the study are:

1- Assess the levels of hsa-miR-590-3p in HCC and OS and identify potential target genes of hsa-miR-590-3p using bioinformatics analysis.
2- Determine the expression of these target genes on the mRNA and protein levels in U2OS, HepG2 and SNU449 using RT-PCR and western blotting, respectively.
3- Determine the expression and localization of hsa-miR-590-3p in relation to its target gene, SOX2, in U2OS and SNU449 using ISH-ICC.

2. Materials and Methods

2.1. In silico Analysis

Prediction of potential downstream target genes of hsa-miR-590-3p was carried out using computational analysis via two approaches: primary screening and function prediction. For the first approach, five databases were used (TargetScan (Agarwal, Bell, Nam, & Bartel, 2015) (http://www.targetscan.org), miRanda-mirSVR (Betel, Koppal, Agius, Sander, & Leslie, 2010) (http://www.microrna.org), miRDB (Wong & Wang, 2015) (http://mirdb.org/), miRTarBase (Chou et al., 2018) (http://mirtarbase.mbc.nctu.edu.tw/php/index.php) and Diana Tools (Paraskevopoulou et al., 2013) (http://diana.imis.athena-innovation.gr)) to obtain a dataset of potential downstream target genes of hsa-miR-590-3p from each database. For the second approach, we used Functional Assignment of MicroRNAs via Enrichment (FAME) software (Ulitsky, Laurent, & Shamir, 2010) (http://acgt.cs.tau.ac.il/fame/index.html) to predict the potential functions of hsa-miR-590-3p through the prediction of the potential functions of its potential downstream target genes. Cancer pathogenesis related functions were chosen, giving rise to a list of genes. These genes were cross-linked with list of the first approach. The Human Protein Atlas (Thul et al., 2017) (http://www.proteinatlas.org/) and previous literature were utilized to narrow our search for potential downstream target genes of hsa-miR-590-3p.
More genes were chosen as potential downstream target genes of hsa-miR-590-3p by aligning their mRNA against hsa-miR-590-3p using miRanda-mirSVR (Betel et al., 2010) (http://www.microrna.org). The mRNA and protein expression of these genes were assessed computationally using The Human Protein Atlas (Thul et al., 2017) (http://www.proteinatlas.org/) and Expression Atlas (Papatheodorou et al., 2018) (http://www.ebi.ac.uk/gxa), respectively.

2.2. Cell Culture

The human hepatocellular carcinoma cell lines used are HepG2 and SNU449. HepG2 is an early-stage liver cancer cell line while SNU449 is an HBV-infected intermediate stage liver cancer cell line. Both cell lines were a kind gift from Dr. Mehmet Ozuturk at the Department of Molecular Biology and Genetics, Bilkent University, Turkey. The Osteosarcoma cell line used is U2OS. U2OS cell line was a kind gift from Dr. Andreas Kakarougkas at the Department of Biology, The American University in Cairo, Egypt. The HCC cell lines and the OS cell line were cultured in RPMI 1640 (Lonza, USA) and DMEM (Lonza, USA) media, respectively, supplemented with 10% FBS (Gibco, USA) and 5% penicillin-streptomycin antibiotic (Lonza, USA). Cells were maintained at 37°C and 5% CO₂ in a humidified atmosphere. Cells were passaged and used for experiments during their logarithmic growth phase. Cells were observed under the inverted microscope (Olympus IX70, USA).

2.3. Viable Cell Count

The trypan blue dye exclusion method was used to detect the viable cell count. An aliquot of the cells was mixed with an equal volume of 0.4% trypan blue (Sigma, USA). An aliquot of this mixture was loaded onto a hemocytometer chamber (Hausser Scientific, USA). The number of the viable cells was counted in the four outer squares of the chamber. The following equation was used to determine the viable cell count per milliliter: [viable cell count (viable cells/ml) = number of viable cells x dilution factor x 10,000].
2.4. Total RNA Extraction, Quantification and Integrity Analysis

Total RNA was extracted from U2OS, HepG2 and SNU449 cells using Trizol reagent (Invitrogen, USA) following the manufacturer’s protocol. RNase free conditions were maintained through the extraction procedure and the extracted RNA was resuspended in DEPC-treated water. DEPC-treated water was prepared by dissolving 1ml DEPC (SERVA Electrophoresis, Germany) in 1000ml distilled water that are left overnight at 37°C and autoclaved the next day. SPECTROstar Nano (BMG LABTECH, Germany) was used to quantify the extracted RNA at wavelength 260nm and assess its purity using 260/280 ratio at wavelengths 260nm and 280nm. RNA integrity was assessed through assessing the rRNA bands on 2% agarose (Bioline, UK) integrity gels.

2.5. cDNA Synthesis and Semi-Quantitative Reverse Transcription – Polymerase Chain Reaction

Total RNA (0.5ug) was used to synthesize cDNA using the RevertAid First Strand cDNA synthesis kit (ThermoScientific, USA) according to the manufacturer’s protocol. mRNA expression was determined using semi-quantitative RT-PCR. MyTaq Red DNA Polymerase (Bioline, UK) was used to perform the PCR reactions using 1ul cDNA per reaction. GAPDH was used as an endogenous control. Specific primers (Invitrogen, USA) were designed using Primer3 (Untergasser et al., 2012) (http://primer3.ut.ee) for each gene. PCR conditions used were the same among the genes except for the annealing temperatures and the number of cycles. PCR conditions are as following: Step1: initiation at 94°C for 3 minutes, Step 2: denaturation at 94°C for 30 seconds, annealing at specific temperatures for each primer for 30 seconds, extension at 72°C for 45 seconds; step 2 is repeated for a specific number of cycles for each primer and Step 3: final extension at 72°C for 10 minutes. Primer sequences, annealing temperatures, number of cycles and PCR amplicon sizes are listed in Table 3. Some primers results into two amplicon sizes, this is due to alternative splicing. PCR products were assessed using agarose gel electrophoresis with the agarose percentage depending on the amplicon size. Amplicon sizes between 100-300bp, 2.5% agarose gels were used and for amplicon sizes between
300-500bp, 2% agarose gels were used and for amplicon sizes larger than 500bp, 1.5% agarose gels were used. The PCR products were visualized using Gel Doc EZ Imager (Bio-Rad, USA).

Table 3. Primers used in Semi-quantitative RT-PCR.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer Sequence</th>
<th>Number of cycles</th>
<th>Annealing Temperature (°C)</th>
<th>Amplicon Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>F: 5'-CCACCCATGGCAATTCCATGGCA-3' R: 5'-TCTAGAGCGGTCAGGTCACC-3'</td>
<td>27</td>
<td>60.5</td>
<td>598</td>
</tr>
<tr>
<td>SOX2</td>
<td>F: 5'-TTACCCTTCCCTCCACCTCC-3' R: 5'-CCTCCATTTCCCTCCATT-3'</td>
<td>35</td>
<td>57</td>
<td>252</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>F: 5'-CCTTCTTCTCCCAATACACATCTCC-3' R: 5'-TTCGCGCTCTTCTCTCATC-3'</td>
<td>30</td>
<td>58</td>
<td>432</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>F: 5'-CAGTGTCAGGACACCAGAT-3' R: 5'-GAAAGCTGAGTGGTGTCACC-3'</td>
<td>30</td>
<td>60</td>
<td>416</td>
</tr>
<tr>
<td>Vimentin</td>
<td>F: 5'-GAACGCCAGATGCGTGAAATG-3' R: 5'-CCAGAGGAGTGATGGTCC-3'</td>
<td>27</td>
<td>60</td>
<td>280</td>
</tr>
<tr>
<td>FOXA2</td>
<td>F: 5'-CTCTGACACGGGTAAATCCA-3' R: 5'-CCTGCAACAGAGGAT-3'</td>
<td>40</td>
<td>55</td>
<td>270</td>
</tr>
<tr>
<td>VCAN</td>
<td>F: 5'-GTTGGGTCTCCAATTCTCGAT-3' R: 5'-GGTTGGGTCTCCAATTCTCGAT-3'</td>
<td>35</td>
<td>55</td>
<td>255</td>
</tr>
<tr>
<td>TMEM33</td>
<td>F: 5'-GGTGTGGGGGAATAATGTGC-3' R: 5'-TTCCAATCATGCAACGGTAA-3'</td>
<td>30</td>
<td>54</td>
<td>172</td>
</tr>
<tr>
<td>DCLRE1A</td>
<td>F: 5'-AACATGTTGCTGTTGCTGAA-3' R: 5'-TTCCTGCAACAGAGGAT-3'</td>
<td>35</td>
<td>57</td>
<td>150</td>
</tr>
<tr>
<td>ERCC5</td>
<td>F: 5'-CAGACACAGCTCCGAATCCA-3' R: 5'-TTCCTGCTCTTTCTCGAT-3'</td>
<td>35</td>
<td>54</td>
<td>209</td>
</tr>
<tr>
<td>UVRAG</td>
<td>F: 5'-GAGCCCTGCTCTTTGGAATGTC-3' R: 5'-TCATGGCAGAAGACTGAC-3'</td>
<td>35</td>
<td>58</td>
<td>179</td>
</tr>
<tr>
<td>NPHP1</td>
<td>F: 5'-TGCCAAGAGGAAATGGAAGTC-3' R: 5'-CACTCCATGGGTGGGCT-3'</td>
<td>35</td>
<td>52</td>
<td>167</td>
</tr>
<tr>
<td>SMC6</td>
<td>F: 5'-AGAAGCTTCTGCTCGTGAGGT-3' R: 5'-TCATGTTTGGCTGAGTCAC-3'</td>
<td>30</td>
<td>56</td>
<td>208</td>
</tr>
<tr>
<td>DLG1</td>
<td>F: 5'-CCAGGCCAGTTACACAGAT-3' R: 5'-TTCCTGCTCCTCTGCTCTCT-3'</td>
<td>30</td>
<td>57</td>
<td>208 &amp; 211</td>
</tr>
<tr>
<td>CX3CL1</td>
<td>F: 5'-TCTGGCAATCTGCAATGC-3' R: 5'-ACCACAGACTGCTCGATGTC-3'</td>
<td>32</td>
<td>57</td>
<td>213 &amp; 334</td>
</tr>
<tr>
<td>BRIP1</td>
<td>F: 5'-AATACCAAGGAAGCAGACTCTG-3' R: 5'-CAGAAGACAGGCAGTTTCA-3'</td>
<td>32</td>
<td>55</td>
<td>242</td>
</tr>
<tr>
<td>HIPK2</td>
<td>F: 5'-CAGTGTCAGTCTCCCTCAA-3' R: 5'-TGAGGCTCTGACTAAGCTG-3'</td>
<td>35</td>
<td>57</td>
<td>191</td>
</tr>
<tr>
<td>DYRK2</td>
<td>F: 5'-TTCCAAGAGCTGCCACATC-3' R: 5'-CCTCAAGAAACTGTTAACAGT-3'</td>
<td>35</td>
<td>55</td>
<td>194</td>
</tr>
<tr>
<td>MLH3</td>
<td>F: 5'-CAATGTCCTGCTTCCATC-3' R: 5'-GAAATGGAAGGTGGTCATGAG-3'</td>
<td>30</td>
<td>57</td>
<td>592</td>
</tr>
<tr>
<td>FANCF</td>
<td>F: 5'-CGGTCAGATGCTAGAGAAGA-3' R: 5'-GCAGGCTAGTCATCAAGTCAA-3'</td>
<td>30</td>
<td>60</td>
<td>360</td>
</tr>
<tr>
<td>RAD21</td>
<td>F: 5'-TGGGTTGTGTTGTTGTTGCTG-3' R: 5'-TCAAGAGGCGTCAATTGTT-3'</td>
<td>30</td>
<td>57</td>
<td>334</td>
</tr>
</tbody>
</table>

*F: Forward * R: Reverse *bp: Basepair
2.6. Total Protein Extraction and Quantification

Total Protein was extracted from U2OS, HepG2 and SNU449 cells using 1X lammeali lysis buffer (50mM Tris/HCl pH 6.8, 2% SDS and 10% glycerol) supplemented with 1X Halt protease inhibitor cocktail (ThermoScientific, USA). ThermoScientific Pierce BCA protein assay kit (ThermoScientific, USA) was used to quantify the total extracted protein according to the manufacturer’s protocol.

2.7. Western Blotting

Total protein (30ug) was used to perform western blotting. Volume equivalent to 30ug was mixed with 4X Lammeali loading dye (240mM Tris/HCl pH 6.8, 8% SDS, 40% glycerol, 0.04% bromophenol blue and 5% freshly added β-mercaptoethanol) and loaded on 10% SDS-Polyacrylamide gel. After separation of the protein on the gel, the protein is blotted onto a nitrocellulose membrane (ThermoScientific, USA) at 150 volts for 90 minutes. The membrane was blocked using 5% non-fat dry milk in 1X TBST (0.01% Tween 20 in 1X TBS buffer) for 1 hour at room temperature. Then the membrane was incubated with the primary antibody (see below) diluted in 5% non-fat dry milk in 1X TBST overnight at 4°C. The next day the membrane was washed 3 times using 1X TBST and incubated with the secondary antibody (see below) diluted in 5% non-fat dry milk in 1X TBST for 1 hour at room temperature. Then the membrane was washed 3 times using 1X TBST. BCIP/NBT Phosphatase colorimetric substrate (KPL, USA) was added. Upon the development of the violet bands, distilled water was added to stop the reaction. GAPDH and β-Tubulin were used as endogenous controls.

Primary antibodies used: anti-GAPDH (1:10,000, ab8245, Abcam, UK), anti- β-Tubulin (1:20,000, T7816, Sigma, USA), anti-SOX2 (1:2000, PA1-16968, ThermoScientific, USA) and anti-Vimentin (1:1000, ab8978, Abcam, UK). Secondary antibodies used: ReverseAP Phosphatase labeled Goat anti-Mouse IgG (H+L) Conjugate (1:20,000, 4751-1806, KPL, USA) and ReverseAP Phosphatase labeled Goat anti-Rabbit IgG (H+L) Conjugate (1:10,000, 4751-1516, KPL, USA).
2.8. *In situ* Hybridization – Immunocytochemistry (ISH-ICC)

For the co-detection of hsa-miR-590-3p and one of its downstream target genes (SOX2), ISH-ICC was carried out on U2OS and SNU449 cells. Cells were seeded into 24-well cell culture plates and incubated till 70% confluency was reached. Fixation of the cells was carried out using 4% formaldehyde and 100% methanol for U2OS and SNU449 respectively. For the ISH, the cells were incubated with DIG-labeled probes for miR-590-3p (hsa-miR-590-3p miRCURY LNA Detection probe, Exiqon, Denmark), U6 (as positive control probe) and scrambled probes (as a negative control) (miRCURY LNA microRNA Detection ISH Buffer and Controls kits, Exiqon, Denmark) for 1 hour at 54°C. Blocking was done using blocking solution rich in 7.5% BSA Fraction V (Gibco, USA) and then the cells were incubated with anti-DIG phosphatase labeled antibody (1:800, 11 093 274 910, Roche, Germany) for 1 hour at room temperature. Cells were then incubated with BCIP/NBT Phosphatase colorimetric substrate ((KPL, USA) in the dark for 2 hours at room temperature. For ICC, blocking was performed again and the cells were incubated with the primary antibody (see below) for 1.5 hours at room temperature. Followed by washes using the blocking solution and the incubation with the secondary antibody (see below) in the dark for 1 hour at room temperature. Then the cells were stained with DAPI (1:1000 in PBS, KPL, USA) and visualized under the inverted microscope (Olympus IX70, USA).

Primary antibody used: Anti-SOX2 (1:250, PA1-16968, ThermoScientific, USA). Secondary antibody used: Goat anti-rabbit IgG (H+L) DyLight 488 Conjugated (1:250, 35552, ThermoScientific, USA).
2.9. Statistical Analysis

For the PCR and western blotting analysis, the obtained bands were quantified and normalized against an endogenous control using Image J Software (Schindelin et al., 2012) (https://imagej.nih.gov/ij/). Data is presented as mean ± standard deviation (SD) from three independent experiments. All statistical comparisons were done using Prism GraphPad 7.0 (Motulsky, 1999) (http://www.graphpad.com/). To analyze the difference between multiple experimental groups with a single variable, one-way ANOVA (with Bonferroni post-test) was used. P-values less than 0.05 are considered significant (* P-value <0.05, ** P-value <0.01 and *** P-value <0.001).

3. Results

3.1 Bioinformatic Analysis to assess the levels of hsa-miR-590-3p in HCC and OS and to Predict Potential Downstream Target Genes of hsa-miR-590-3p

3.1.1. Assessing the levels of hsa-miR-590-3p in HCC and OS

Several bioinformatics tools were utilized to assess the levels of hsa-miR-590-3p in HCC and OS tissues or cell lines. Despite the available experimental studies, we were unable to obtain any results for the levels of hsa-miR-590-3p in HCC and OS via in silico analysis.

3.1.2. Primary Screening of Potential Targets of hsa-miR-590-3p (First Approach)

To predict the potential downstream target genes of hsa-miR-590-3p, five databases (TargetScan, miRanda-mirSVR, miRDB, miRTarBase and Diana Tools) were used. TargetScan predicts a miRNA’s target genes through the conserved sites of these genes that match the miRNA’s seed region (Lewis, Burge, & Bartel, 2005). 8,611 potential target genes of hsa-miR-590-3p were obtained from TargetScan (Appendix A). Table 4 represents some of the top downstream target genes with the best total context++ score obtained from TargetScan (“TargetScan,” accessed May 2017). The total context++ score
is obtained from the sum of the contribution of fourteen features assessed in the database. MiRanda-miRsvr predicts potential target genes of miRNAs through a two-step strategy. First, the program goes through a local alignment of the miRNA sequence against mRNA sequences and produces a score based on the complementarity through A:U and G:C matches. Second, it uses the high-scoring alignments, meaning they passed a certain threshold, to calculate their thermodynamic stability (Betel et al., 2010). 21,123 potential downstream target genes of hsa-miR-590-3p were obtained from miRanda-miRsvr. MiRDB uses the bioinformatics tool, MirTarget. Through the analysis of thousands of miRNA-target interactions obtained from high-throughput sequencing experiments, MirTarget was developed (X. Wang, 2016). 1,590 potential target genes of hsa-miR-590-3p were obtained from miRDB (Appendix B). Table 5 represents the top ranked downstream target genes with the best target score obtained from miRDB (“miRDB,” accessed May 2017). The target score is assigned by the computational target prediction algorithm and is validated experimentally. MiRTarBase is a database based on the collection of miRNA-target interactions (MTIs) from the previous literature and validating these interactions experimentally using next-generation sequencing (NGS), microarray, western blotting and reporter assay (Chou et al., 2018). 447 potential target genes of hsa-miR-590-3p were obtained from miRTarBase (Appendix C). Table 6 represents the top ranked downstream target genes validated through NGS, which is considered the most reliable validation method, obtained from miRTarBase (“miRTarBase,” accessed May 2017). Diana TarBase is a database that is established using specific and high throughput experiments to predict miRNA-gene interactions (Paraskevopoulou et al., 2013). 4,576 potential target genes of hsa-miR-590-3p were obtained from Diana TarBase (Appendix D). Table 7 represents the top ranked downstream target genes with the best prediction score (miTG score) obtained from Diana Tools (“Diana Tools,” accessed May 2017). The prediction score (miTG score) is based on experimental validation using TarBase and TargetScan prediction. All potential downstream target genes of hsa-miR-590-3p from all 5 databases were placed in a pivot table and a list of 362 common genes was obtained (Figure 3, Appendix E).
Table 4. Some of the Top Potential Downstream Target Genes of hsa-miR-590-3p obtained from TargetScan based on Total Context++ Score.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Gene Name</th>
<th>Cumulative Weighted context++ score</th>
<th>Total context++ score</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOP14</td>
<td>NOP14 nucleolar protein</td>
<td>-0.01</td>
<td>-0.01</td>
</tr>
<tr>
<td>CFHR3</td>
<td>Complement factor H-related 3</td>
<td>-0.01</td>
<td>-0.01</td>
</tr>
<tr>
<td>DMRTA2</td>
<td>DMRT-like family A2</td>
<td>-0.01</td>
<td>-0.01</td>
</tr>
<tr>
<td>GPATCH2</td>
<td>G patch domain containing 2</td>
<td>-0.01</td>
<td>-0.01</td>
</tr>
<tr>
<td>CDCA7L</td>
<td>Cell division cycle associated 7-like</td>
<td>-0.01</td>
<td>-0.01</td>
</tr>
<tr>
<td>DMRT1</td>
<td>Doublesex and mab-3 related transcription factor 1</td>
<td>-0.01</td>
<td>-0.01</td>
</tr>
<tr>
<td>TMEM41A</td>
<td>Transmembrane protein 41A</td>
<td>-0.01</td>
<td>-0.01</td>
</tr>
</tbody>
</table>

Table 5. The Top Ranked Potential Downstream Target Genes of hsa-miR-590-3p obtained from miRDB based on Target Score.

<table>
<thead>
<tr>
<th>Target Score</th>
<th>Gene Symbol</th>
<th>Gene Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>RIMKLB</td>
<td>Ribosomal modification protein rimK-like family member B</td>
</tr>
<tr>
<td></td>
<td>LTN1</td>
<td>Listerin E3 ubiquitin protein ligase 1</td>
</tr>
<tr>
<td></td>
<td>RYBP</td>
<td>RING1 and YY1 binding protein</td>
</tr>
<tr>
<td></td>
<td>ZDHHC21</td>
<td>Zinc finger, DHHC-type containing 21</td>
</tr>
<tr>
<td></td>
<td>TWF1</td>
<td>Twinfilin actin-binding protein 1</td>
</tr>
<tr>
<td></td>
<td>RBM12B</td>
<td>RNA binding motif protein 12B</td>
</tr>
<tr>
<td></td>
<td>JMJD1C</td>
<td>Jumonji domain containing 1C</td>
</tr>
</tbody>
</table>
Table 6. The Top Ranked Potential Downstream Target Genes of hsa-miR-590-3p obtained from miRTarBase based on the most reliable validation method.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Validation Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPATCH8</td>
<td></td>
</tr>
<tr>
<td>PARP16</td>
<td></td>
</tr>
<tr>
<td>CHAF1A</td>
<td></td>
</tr>
<tr>
<td>TMEM259</td>
<td></td>
</tr>
<tr>
<td>MTF1</td>
<td></td>
</tr>
<tr>
<td>SKI</td>
<td></td>
</tr>
<tr>
<td>NDUFB5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sequencing</td>
</tr>
</tbody>
</table>

Table 7. The Top Ranked Potential Downstream Target Genes of hsa-miR-590-3p obtained from Diana Tools Based on miTG Score.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>miTG Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLP1</td>
<td>0.99999843</td>
</tr>
<tr>
<td>LARP4</td>
<td>0.999997473</td>
</tr>
<tr>
<td>MBNL1</td>
<td>0.999991713</td>
</tr>
<tr>
<td>WASL</td>
<td>0.9999879</td>
</tr>
<tr>
<td>NAMPT</td>
<td>0.999983784</td>
</tr>
<tr>
<td>ZNF738</td>
<td>0.999975655</td>
</tr>
<tr>
<td>KIAA0087</td>
<td>0.999962665</td>
</tr>
</tbody>
</table>
3.1.3. Function Prediction of Potential Targets of hsa-miR-590-3p (Second Approach)

A miRNA’s function is anticipated through its post-transcriptional regulation of its downstream target genes. In order to get further insight of hsa-miR-590-3p’s function, we performed the functional prediction analysis of its downstream target genes using FAME software. FAME analysis is based on a collection of miRNA-pathways and miRNA-process association that has been verified experimentally (Ulitsky et al., 2010). FAME identified many functions and the downstream targets genes of hsa-miR-590-3p with these functions. Cancer pathogenesis related functions were chosen (Table 8). These functions include response to DNA damage stimulus, DNA repair, cell-cell adhesion,
nucleotide-excision repair and DNA damage response and signal transduction (Figure 4).
From these functions a list of thirty-four genes were obtained.

Table 8. The Chosen Functions of the Potential Downstream Target Genes of hsa-miR-590-3p obtained from FAME Software.

<table>
<thead>
<tr>
<th>Function</th>
<th>FAME p-value</th>
<th>FAME Enrichment factor</th>
<th>Genes linked to Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Response to DNA Damage Stimulus</td>
<td>0.0198</td>
<td>1.51</td>
<td>ANKRD17, BRIP1, CHES1, DCLRE1A, DYRK2, EPC2, ERCC5, ESCO2, FANCF, HIPK2, MAPK1, MLH3, MYO6, POLS, RAD21, RAD23B, SFPQ, SMC6, UBE2N and UVRAG</td>
</tr>
<tr>
<td>DNA Repair</td>
<td>0.0251</td>
<td>1.62</td>
<td>ANKRD17, BRIP1, CHES1, DCLRE1A, DYRK2, EPC2, ERCC5, ESCO2, FANCF, MLH3, POLS, RAD21, RAD23B, SFPQ, SMC6, UBE2N and UVRAG</td>
</tr>
<tr>
<td>Cell-cell Adhesion</td>
<td>0.0361</td>
<td>1.5</td>
<td>CD164, CLDN22, CLSTN1, CTNND2, CX3CL1, DLG1, DSC3, FAT3, MSN, NLGN1, NPHP1, PCDH19, PKHD1 and VCAM1</td>
</tr>
<tr>
<td>Nucleotide-Excision Repair</td>
<td>0.0382</td>
<td>3.01</td>
<td>DCLRE1A, ERCC5 and RAD23B</td>
</tr>
<tr>
<td>DNA Damage Response and Signal Transduction</td>
<td>0.0439</td>
<td>1.81</td>
<td>BRIP1, CHES1, DYRK2, HIPK2 and MYO6</td>
</tr>
</tbody>
</table>
3.1.4. Potential Targets of hsa-miR-590-3p using The Human Protein Atlas

Thirty-two genes from the function prediction approach were found in the list from the primary screening approach. These thirty-two genes were further analyzed through the previous literature and The Human Protein Atlas (Thul et al., 2017). Eleven genes were chosen based on the results obtained from The Human Protein Atlas (Figure 5) and three genes were chosen based on previous literature as these genes’ protein information are not provided through The Human Protein Atlas. The fourteen genes are BRIP1, CX3CL1, DCLRE1A, DLG1, DYRK2, ERCC5, FANC, HIPK2, MLH3, NPHP1, RAD21, SMC6, TMEM33 and UVRAG. The genes were categorized according to the functions they share (Figure 6).
Figure 5. Protein Expression of Potential Targets of hsa-miR-590-3p in Normal versus Cancerous Liver Tissue using The Human Protein Atlas. Illustration of the protein expression of eleven genes chosen for further analysis in normal liver tissue versus cancerous liver tissue. Scale: 0- Not detected, 1-Low, 2-Medium and 3-High.

Figure 6. Categorization of the Fourteen Genes Based on the Function they Share. Figure generated using Microsoft PowerPoint.
3.2. mRNA Expression of Potential Downstream Target Genes of hsa-miR-590-3p

The mRNA levels of the potential downstream target genes of hsa-miR-590-3p were analyzed using RT-PCR. Expression levels were normalized against GAPDH as an endogenous control using Image J and compared to each other using Prism Graphpad.

3.2.1. mRNA Expression of the Fourteen Potential Targets of hsa-miR-590-3p in HCC cell lines

The mRNA levels of the fourteen potential downstream target genes of hsa-miR-590-3p in HCC cell lines, HepG2 and SNU449, were analyzed using RT-PCR. mRNA expression levels of all genes except CX3CL1 showed no significant difference between HepG2 and SNU449 (Figure 7). CX3CL1 mRNA expression is higher in HepG2 compared to SNU449 with a (P<0.01) (Figure 8).

3.2.2. mRNA Expression of CX3CL1 in U2OS

The mRNA expression level of CX3CL1 in the OS cell line, U2OS, was measured using RT-PCR. CX3CL1 mRNA expression in U2OS showed no significant difference compared to HepG2 and higher expression compared to SNU449 with a (P<0.01) (Figure 8).
Figure 7. mRNA Expression of Potential Targets of hsa-miR-590-3p in HepG2 and SNU449 using RT-PCR. Images are categorized according to the function of the genes. GAPDH is used as an endogenous control for all genes. RT-PCR band intensities were measured using Image J and normalized against GAPDH as an endogenous control and statistically analyzed using Prism GraphPad. **A&F.** Functions: Response to DNA damage stimulus and DNA repair. mRNA expression of UVRAG, SMC6, MLH3, FANCF and RAD21. No statistically significant difference was observed between both cell lines (P>0.05). **B&G.** Functions: Response to DNA damage stimulus and DNA damage response and signal transduction. mRNA expression of BRIP1, DYRK2 and HIPK2. No statistically significant difference was observed between both cell lines (P>0.05). **C&H.** Functions: Response to DNA damage stimulus, DNA Repair and nucleotide-excision repair. mRNA expression of ERCC5 and DCLRE1A. No statistically significant difference was observed between both cell lines (P>0.05). **D&I.** mRNA expression of TMEM33, a trans-membrane protein. No statistically significant difference was observed between both cell lines (P>0.05). **E&J.** Functions: Cell-cell adhesion. mRNA expression of DLG1 and NPHP1. No statistically significant difference was observed between both cell lines (P>0.05). Negatives were carried out for all experiments. Results are a representation of three independent experiments.
Figure 8. mRNA Expression of CX3CL1 in U2OS, HepG2 and SNU449 using RT-PCR. A. Agarose gel electrophoresis results for mRNA expression of CX3CL1, a gene involved with cell-cell adhesion, and GAPDH as an endogenous control in U2OS, HepG2 and SNU449. Negatives were carried out for all experiments. Only one amplicon size at 334bp was detected. B. RT-PCR band intensities were measured using Image J and normalized against GAPDH and statistically analyzed using Prism GraphPad. mRNA expression of CX3CL1 in U2OS, HepG2 and SNU449. U2OS and HepG2 show comparable expression (P>0.05) while SNU449 shows decreased expression compared to U2OS and HepG2 (P<0.01). Results are a representation of three independent experiments.

3.3. Bioinformatic Analysis to Validate Other Potential Downstream Target Genes of hsa-miR-590-3p

3.3.1. Alignment of Potential Targets of hsa-miR-590-3p using miRanda-mirSVR

New genes were identified as potential downstream target genes of hsa-miR-590-3p using miRanda-mirSVR by aligning hsa-miR-590-3p against the mRNA of specific genes. These genes include E-cadherin, N-cadherin, SOX2 and FOXA2. hsa-miR-590-3p has one binding site on E-cadherin mRNA at position 1432 (Figure 9A) and one binding site on N-cadherin mRNA at position 586 (Figure 9B), three binding sites on SOX2
mRNA at positions 606, 640 and 658 (Figure 9C) and one binding site on FOXA2 mRNA at position 817 (Figure 9D).

Figure 9. Alignment of the mRNA of Potential Targets of hsa-miR-590-3p against hsa-miR-590-3p using miRanda-mirSVR. A. Binding site of hsa-miR-590-3p on E-cadherin mRNA at position 1432. B. Binding site of hsa-miR-590-3p on N-cadherin mRNA at position 586. C. Binding sites of hsa-miR-590-3p on SOX2 mRNA at positions 606, 640 and 658. D. Binding site of hsa-miR-590-3p on FOXA2 mRNA at position 817.
3.3.2. mRNA and Protein Expression of Potential Targets of hsa-miR-590-3p using The Human Protein Atlas and Expression Atlas

The mRNA and protein expression of E-cadherin, N-cadherin, SOX2 and FOXA2 were assessed computationally using The Human Protein Atlas and Expression Atlas, respectively. The Human Protein Atlas shows the expression in U2OS and HepG2 while Expression Atlas compares the expression between the HCC cell lines, HepG2 and SNU449, and the OS cell lines, U2OS, MG-63 and Saos-2. Also, Vimentin was assessed as an epithelial-mesenchymal transition (EMT) marker and VCAN as a downstream target gene of FOXA2. The results are illustrated in figures 10 and 11.
Figure 10. mRNA Expression of Potential Targets of hsa-miR-590-3p in U2OS and HepG2 using The Human Protein Atlas. A. mRNA expression of E-cadherin, N-cadherin and Vimentin in U2OS and HepG2. B. mRNA expression of SOX2 in U2OS and HepG2. C. mRNA expression of FOXA2 and VCAN in U2OS and HepG2.
Figure 11. Protein Expression of Potential Targets of hsa-miR-590-3p in OS and HCC cell lines using Expression Atlas. A. Protein expression of E-cadherin, N-cadherin and Vimentin in HepG2 and SNU449. B. Protein expression of E-cadherin, N-cadherin and Vimentin in U2OS, MG-63 and Saos-2. C. Protein Expression of SOX2 in U2OS, MG-63 and Saos-2. D. Protein expression of FOXA2 and VCAN in HepG2 and SNU449. E. Protein expression of FOXA2 and VCAN in U2OS, MG-63 and Saos-2.

3.4. mRNA Expression of E-cadherin and N-cadherin as Potential Targets of hsa-miR-590-3p using RT-PCR and mRNA expression of Vimentin as a Mesenchymal Marker

The mRNA levels of E-cadherin, N-cadherin and Vimentin in U2OS, HepG2 and SNU449 were analyzed using RT-PCR. Expression levels were normalized against GAPDH as an endogenous control using Image J and plotted using Prism Graphpad. U2OS and SNU449 show comparable E-cadherin mRNA expression (P>0.05) while HepG2 shows increased E-cadherin mRNA expression compared to U2OS and SNU449 (P<0.01) (Figure 12). No Significant difference was observed between the N-cadherin mRNA expression in U2OS, HepG2 and SNU449 (P>0.05) (Figure 12). U2OS and HepG2 show comparable Vimentin mRNA expression (P>0.05) while SNU449 shows increased Vimentin mRNA expression compared to U2OS and HepG2 (P<0.01) (Figure 12).
Figure 12. mRNA Expression of E-cadherin, N-cadherin and Vimentin in U2OS, HepG2 and SNU449 using RT-PCR. GAPDH was used as an endogenous control. A. Agarose gel electrophoresis results for mRNA expression of E-cadherin, N-cadherin and Vimentin U2OS, HepG2 and SNU449 respectively. B. RT-PCR band intensities were measured using Image J and normalized against GAPDH and statistically analyzed using Prism GraphPad. U2OS and SNU449 show comparable E-cadherin expression (P>0.05) while HepG2 shows increased expression compared to U2OS and SNU449 (P<0.01). No significant difference was observed between the N-cadherin mRNA expression in U2OS, HepG2 and SNU449 (P>0.05). U2OS and HepG2 show comparable Vimentin expression (P>0.05) while SNU449 shows increased expression compared to U2OS and HepG2 (P<0.01). In U2OS, N-cadherin and Vimentin mRNA expression are significantly higher than E-cadherin (P<0.001). In HepG2, no statistically significant change was observed among all three genes (P>0.05). In SNU449, N-cadherin and Vimentin mRNA expression are significantly higher than E-cadherin (P<0.001). Negatives were carried out for all experiments. Results are a representation of three independent experiments.

3.5. Protein Expression of Vimentin as a Mesenchymal Marker

The protein level of Vimentin in U2OS, HepG2 and SNU449 were analyzed using western blotting. Expression levels were normalized against GAPDH as an endogenous control using Image J and compared to each other using Prism Graphpad. U2OS and SNU449 show comparable Vimentin protein expression (P>0.05) while it is not detected in HepG2 (P<0.001) (Figure 13).
Figure 13. Protein Expression of Vimentin in U2OS, HepG2 and SNU449 using Western Blotting. GAPDH was used as an endogenous control. A. Membrane image for protein expression of Vimentin in U2OS, HepG2 and SNU449. B. Western blotting band intensities were measured using Image J and normalized against GAPDH and statistically analyzed using Prism GraphPad. Vimentin protein expression was comparable in U2OS and SNU449 (P>0.05) while it was not detected in HepG2 (P<0.001). Two bands were observed in SNU449 while only one band was observed in U2OS. Negatives were carried out for RT-PCR experiments. Results are a representation of three independent experiments.

3.6. mRNA & Protein Expression of SOX2 as a Potential Target of hsa-miR-590-3p

The mRNA and protein levels of SOX2 in U2OS, HepG2 and SNU449 were analyzed using RT-PCR and western blotting, respectively. Expression levels were normalized against GAPDH and β-tubulin as an endogenous controls using Image J and compared using Prism Graphpad. U2OS and SNU449 show comparable SOX2 mRNA expression (P>0.05) while HepG2 shows decreased SOX2 mRNA expression compared to U2OS and SNU449 (P<0.01) (Figure 14A and 14C). HepG2 and SNU449 show comparable SOX2 protein expression (P>0.05) while U2OS shows decreased SOX2 protein expression compared to HepG2 and SNU449 (P<0.001) (Figure 14B and 14D).
Figure 14. mRNA and Protein Expression of SOX2 in U2OS, HepG2 and SNU449 using RT-PCR and Western Blotting Respectively. GAPDH and β-tubulin were used as endogenous controls. A. Agarose gel electrophoresis results for mRNA expression of SOX2 in U2OS, HepG2 and SNU449. B. Membrane image for protein expression of SOX2 in U2OS, HepG2 and SNU449. C & D. RT-PCR and western blotting band intensities were measured using Image J and normalized against GAPDH and β-tubulin respectively and statistically analyzed using Prism GraphPad. C. mRNA expression of SOX2 in U2OS, HepG2 and SNU449. U2OS and SNU449 show comparable expression (P>0.05) while HepG2 shows decreased expression compared to U2OS and SNU449 (P<0.01). D. Protein expression of SOX2 in U2OS, HepG2 and SNU449. HepG2 and SNU449 show comparable SOX2 protein expression (P>0.05) while U2OS shows decreased expression compared to HepG2 and SNU449 (P<0.001). Negatives were carried out for RT-PCR experiments. Results are a representation of three independent experiments.
3.7. mRNA Expression of FOXA2 as a Potential Target of hsa-miR-590-3p and its Downstream Target Gene VCAN

The mRNA levels of FOXA2 and VCAN in U2OS, HepG2 and SNU449 were analyzed using RT-PCR. Expression levels were normalized against GAPDH as an endogenous control using Image J and compared using Prism GraphPad. No significant difference was observed between the FOXA2 mRNA expression in U2OS, HepG2 and SNU449 (P>0.05) (Figure 15). HepG2 and SNU449 show comparable VCAN mRNA expression (P>0.05) while U2OS shows decreased VCAN mRNA expression compared to HepG2 and SNU449 (P<0.001) (Figure 15).

**Figure 15. mRNA Expression of FOXA2 and VCAN in U2OS, HepG2 and SNU449 using RT-PCR.** GAPDH was used as an endogenous control. **A.** Agarose gel electrophoresis results for mRNA expression of FOXA2 and VCAN in U2OS, HepG2 and SNU449. **B.** RT-PCR band intensities were measured using Image J and normalized against GAPDH and statistically analyzed using Prism GraphPad. No significant difference was observed between the FOXA2 mRNA expression in U2OS, HepG2 and SNU449 (P>0.05). HepG2 and SNU449 show comparable VCAN mRNA expression (P>0.05) while U2OS shows decreased VCAN mRNA expression compared to HepG2 and SNU449 (P<0.001). In U2OS, there is a negative correlation between FOXA2 and VCAN mRNA expression; FOXA2 expression is high while VCAN expression is low (P<0.01). In HepG2 and SNU449, no statistically significant change was observed among
the mRNA levels of FOXA2 and VCAN (P>0.05). Negatives were carried out for all experiments. Results are a representation of three independent experiments.

3.8. Expression and Localization of hsa-miR-590-3p and its Potential Target, SOX2, in U2OS and SNU449 cells

Expression and localization of hsa-miR-590-3p and SOX2 in U2OS and SNU449 were assessed using ISH-ICC (Figures 16 and 17). The signal intensity of hsa-miR-590-3p and the cell fluorescence of SOX2 in each cell line were assessed using Image J and compared using Prism GraphPad. hsa-miR-590-3p signal intensity is higher in SNU449 compared to U2OS (P>0.05) while SOX2 fluorescence is more intense in U2OS compared to SNU449 (P>0.01) (Figure 18). Cells that showed hsa-miR-590-3p signal showed minimal SOX2 fluorescence (red circles, Figures 16 and 17) and cells that did not show hsa-miR-590-3p signal, SOX2 fluorescence was detected (white circles, Figures 16 and 17).
Figure 16. Expression and Localization of hsa-miR-590-3p and SOX2 in U2OS cells.
A. hsa-miR-590-3p signal in U2OS cells under the 20X magnification using ISH (red circle). B. SOX2 fluorescent signal in U2OS cells under the 20X magnification using ICC (white circle). C. DAPI staining of the nucleus. D. Merged image of B and C. E. U6 signal U2OS cells under the 20X magnification using ISH as a positive control (red circles). F. Negative control in U2OS cells under the 20X magnification using ISH using scrambled probes. Scale for A-F: 20µm.
Figure 17. Expression and Localization of hsa-miR-590-3p and SOX2 in SNU449 cells. A. hsa-miR-590-3p signal in SNU449 cells under the 20X magnification using ISH (red circle). B. SOX2 florescent signal in SNU449 cells under the 20X magnification using ICC (white circle). C. DAPI staining of the nucleus. D. Merged image of B and C. E. U6 signal SNU449 cells under the 20X magnification using ISH as a positive control (red circles). F. Negative control in SNU449 cells under the 20X magnification using ISH using scrambled probes. Scale for A-F: 20µm.
Figure 18. Graphical Representation of the Expression of hsa-miR-590-3p and SOX2 in U2OS and SNU449 cells. The cell fluorescence of SOX2 and the signal intensity of hsa-miR-590-3p were assessed using Image J and compared using Prism GraphPad. **A.** Representation of hsa-miR-590-3p signal intensity. hsa-miR-590-3p signal intensity is higher in SNU449 compared to U2OS (P>0.05). **B.** Representation of the corrected total cell fluorescence (CTCF) of SOX2. SOX2 fluorescence is higher in U2OS compared to SNU449 (P>0.01). Results are a representation of three independent experiments.
4. Discussion

Hepatocellular carcinoma and osteosarcoma are two very aggressive malignant tumors with poor prognosis. Both tumors are usually diagnosed during stage III or even worse during stage IV of the cancer when most medications and surgical interventions are not efficient. Therefore, understanding the cancer progression and pathogenesis is essential.

Twenty years ago, miRNAs were discovered, opening the door for further understanding of cancer pathogenesis. Several studies addressed miRNAs (miR-17, miR-21, miR-22, miR-26, miR-29b, miR-122, miR-135a, miR-146a, miR-151, miR-181b, miR-221/222, miR-224 miR-233, miR-338-3p, miR-491 and miR-500) in relation to HCC and various liver diseases (HCV, HBV and non-alcoholic fatty liver disease) (Abba et al., 2014; Kerr, Korenblat, & Davidson, 2011; Nana-Sinkam, 2011; Takahashi, Yan, Wen, & Patel, 2013; Wittmann & Jäck, 2010; Yamamoto et al., 2009). MiRNAs have been identified in the serum and liver tissues of HCC patients; some were up-regulated while others were down-regulated. Other studies addressed miRNAs (miR-16, miR-100-5p, miR-221-3p, miR-29b-1-5p, miR-125b-1-3p, miR-29a-5p, miR-370-3p, miR-299-5p, miR-181a-5p, miR-181c-5p, miR-223-3p, miR-342-3p and miR-378a-3p) in relation to osteosarcoma (Andersen, Knudsen, Hager, Hansen, & Tost, 2018; Liang, Gao, Fu, Xu, & Qian, 2013).

hsa-miR-590-3p has been tested previously in relation to glioblastoma multiforme (GBM), an aggressive brain cancer, through tissue specimen and cell lines (Pang et al., 2015). It has also been tested in relation to epithelial ovarian cancer (EOC) through tissue specimen and serum samples (Salem et al., 2018). In GBM tissue specimens and cell lines, hsa-miR-590-3p was significantly down-regulated compared to normal tissues. Unlike GMB, in EOC serum samples and tissue specimens, hsa-miR-590-3p was significantly up-regulated in the cancerous ovarian tissue compared to the normal ovarian tissue. The difference in the expression of one miRNA (hsa-miR-590-3p) in two different types of tumors (GBM and EOC) suggests that hsa-miR-590-3p is tissue specific and can regulate tumor suppressor genes in one tissue type and regulate oncogenes in another.
hsa-miR-590-3p has been previously studied in HCC in tissue specimen and in cell lines (Ge & Gong, 2017; Hongfei, Wenhong, Wentao, Chunyan, & Jing, 2013). hsa-miR-590-3p was found to be significantly down-regulated in cancer tissue compared to normal tissues and significantly up-regulated in three HCC cell lines (HepG2, Hep3B and Huh7). This discrepancy in the reported results may be due to the fact that tissue specimens are heterogeneous and several factors may affect the miRNA’s expression and that cell lines may change their characteristics and in turn their gene expression when they have been in culture for a long time. This discrepancy in the reported results and the lack of reported results concerning the expression levels of hsa-miR-590-3p in OS encouraged us to study hsa-miR-590-3p in HCC and OS and its downstream target genes.

### 4.1. Bioinformatic Analysis to Predict Potential Downstream Target Genes of hsa-miR-590-3p

In the present study, we went through a sequence of bioinformatics analysis to predict potential downstream target genes of hsa-miR-590-3p. For preliminary screening, five databases (TargetScan, miRanda-mirSVR, miRDB, miRTarBase and Diana Tools) were used (Figure 3). For function prediction, we used FAME software (Figure 4). From each approach a list of potential downstream target genes was obtained. Thirty-two genes from the first approach were found in the list from the second approach. Through further in silico analysis using The Human Protein Atlas to assess protein levels of the potential targets of hsa-miR-590-3p in normal liver tissue versus cancerous liver tissue, fourteen genes were chosen for further validation using molecular techniques (Figure 5).

### 4.2. Potential Downstream Target Genes of hsa-miR-590-3p

The fourteen genes are BRIP1, CX3CL1, DCLRE1A, DLG1, DYRK2, ERCC5, FANCF, HIPK2, MLH3, NPHP1, RAD21, SMC6, TMEM33 and UVRAG. The genes were categorized according to the functions they share (Figure 6).
UVRAG, SMC6, MLH3, FANCF and RAD21 belong to the first category: response to DNA damage stimulus and DNA repair. UVRAG encodes the UV radiation resistance associated protein. UVRAG activates the Beclin1-PI(3)KC3 complex which promotes autophagy and suppresses the proliferation and tumorigenicity of human colon cancer cells (Kim et al., 2008). SMC6 encodes the structural maintenance of chromosomes 6 protein and is mainly involved in DNA repair (Torres-Rosell et al., 2005). MLH3 is a member of the MutL-homolog family which is involved with DNA mismatch repair genes (Taylor et al., 2006). FANCF, also known as fanconi anemia complementation group F, is a member of fanconi anemia complementation group which are essential in DNA repair (Narayan et al., 2004). RAD21 cohesin complex component is essential for mitotic growth and is involved in the repair of DNA double-strand breaks (Yamamoto et al., 2006).

BRIP1, DYRK2 and HIPK2 belong to the second category: response to DNA damage stimulus and DNA damage response and signal transduction. BRIP1, also known as BRCA1 interacting protein C-terminal helicase 1, forms a complex with BRCA1 that is important in the repair of double-strand breaks (Monteiro et al., 2013). DYRK2, also known as dual specificity tyrosine phosphorylation regulated kinase 2, is a member of a protein kinase family which is involved in cellular growth and development (Yan et al., 2016). HIPK2, also known as homeodomain-interacting protein kinase 2, is a cell growth and apoptosis regulator (Somboek & Hofmann, 2009).

ERCC5 and DCLRE1A belong to the third category: response to DNA damage stimulus, DNA repair and nucleotide-excision repair. ERCC5, known as excision repair cross-complementing 5, encodes a single-strand specific DNA endonuclease which makes a 3’ incision following UV-induced damage in the DNA excision repair (Wang et al., 2016). DCLRE1A, known as DNA cross-link repair 1A, encodes a conserved protein that plays a role in the DNA interstrand cross-links repair (Ma, Huang, & Moran, 2009).

TMEM33, Transmembrane protein 33, is a transmembrane protein involved in endoplasmic reticulum stress responsive events in cancer cells (Sakabe, Hu, Jin, Clarke, & Kasid, 2015). CX3CL1, DLG1 and NPHP1 are involved in cell-cell adhesion. DLG1,
known as human discs large tumor suppressor, regulates cell polarity and proliferation proposing a connection between epithelial organization and cellular growth control (Cavatorta et al., 2017). NPHP1, nephrocystin 1, encodes a protein that interacts with Crk-associated substrate and it is involved in cell division, cell-cell and cell-matrix adhesion (Snoek et al., 2018). CX3CL1, C-X3-C motif chemokine ligand 1 or chemokine fractalkine, has been reported in many epithelial tissues and mediates strong cell adhesion (Gaudin et al., 2011).

The mRNA expression of all these genes was assessed in the HCC cell lines, HepG2 and SNU449. All the genes showed comparable expression between both cell lines except CX3CL1 (Figure 7). HepG2 showed high CX3CL1 mRNA expression compared to SNU449 (Figure 8). HepG2 is an early-stage well-differentiated human HCC cell line while SNU449 is an intermediate stage HCC cell line that explains the high mRNA expression of CX3CL1 in HepG2 and the low expression in SNU449 as high expression of CX3CL1 indicates tighter cell adhesion, thus, no metastasis. U2OS, a human OS epithelial cell line, was also used to assess CX3CL1 mRNA expression. U2OS showed comparable expression compared to HepG2 and high expression compared to SNU449 (Figure 8).

Only one gene, CX3CL1, of the fourteen chosen potential targets of hsa-miR-590-3p showed differential expression between the tested HCC cell lines. Since CX3CL1 is involved in cell-cell adhesion, we decided to choose genes related to that function and validate them as potential downstream target genes of hsa-miR-590-3p but using miRanda-mirSVR. E-cadherin and N-cadherin mRNA were aligned against hsa-miR-590-3p to assess the binding sites of our miRNA on the mRNA of these genes (Figure 9).

4.3. Potential Targets of hsa-miR-590-3p and Cell-cell Adhesion

Cadherins are calcium dependent cell adhesion molecules mainly involved in cell-cell adhesion and cell migration (Nakajima, Doi, & Toyoda, 2004). There are several types of cadherins but in this study we are focusing on E-cadherin (CDH1) and N-cadherin
(CDH2). E-cadherin, known as epithelial cadherin is what holds epithelial cells together. As cancer progresses, cells start to lose their E-cadherin expression and start producing N-cadherin. N-cadherin, known as neural cadherin, increases with the cell’s increased invasiveness potential (Khorram-Manesh, Ahlman, Jansson, & Nilsson, 2002). These molecular changes take place during a process called epithelial-mesenchymal transition (EMT). EMT is a process in which epithelial cells lose their adherent nature and acquire mesenchymal traits, including migration and invasion abilities hence the ability to metastasize to other organs (Zheng et al., 2018) (Figure 19). Among these molecular changes is alteration in Vimentin expression (a mesenchymal marker), which increases as the cancer progresses and the cells become more invasive (Nakajima et al., 2004).

![Diagram of Epithelial-Mesenchymal Transition](image)

**Figure 19. Illustration of Epithelial-Mesenchymal Transition.** The diagram shows the transition of epithelial cells to mesenchymal cells and how they lose their adherent traits and acquire the ability to move and become metastatic during a process known as EMT. E-cadherin is an epithelial marker while N-cadherin and Vimentin are mesenchymal markers. The figure was generated using Lucidchart.

The mRNA expression of E-cadherin, N-cadherin and Vimentin in U2OS and HepG2 was assessed computationally using The Human Protein Atlas. E-cadherin mRNA expression is higher in HepG2 compared to U2OS while N-cadherin mRNA expression is comparable in both cell lines. Vimentin expression was much higher in U2OS compared to HepG2 (Figure 10). The protein expression of E-cadherin, N-cadherin and Vimentin in HCC cell lines, HepG2 and SNU449, and OS cell lines, U2OS, MG-63 and Saos-2 was
assessed computationally using Expression Atlas. MG-63 is heterogeneous OS cell line, exhibiting both mature and immature osteoblasts, giving it its mesenchymal characteristics. Saos-2 is considered an epithelial OS cell line although it contains mature osteoblasts (Pautke et al., 2004). For the HCC cell lines, E-cadherin and N-cadherin protein expression is higher in HepG2 compared to SNU449. As for the OS cell lines, E-cadherin is minimally expressed in U2OS and is not detected in MG-63 and Saos-2. N-cadherin is comparably expressed in all cell lines. Vimentin is comparable in U2OS and SNU449 while it was not detected in HepG2 (Figure 11).

The mRNA expression of E-cadherin, N-cadherin and Vimentin was assessed in HepG2, SNU449 and U2OS using RT-PCR (Figure 12). HepG2 showed high E-cadherin expression, low N-cadherin expression and low Vimentin expression compared to SNU449. Since HepG2 is an early-stage well-differentiated cell line of epithelial origin, it is retaining its epithelial characteristics, hence the increase in the epithelial markers and the decrease in the mesenchymal markers. SNU449 showed low E-cadherin expression, high N-cadherin expression and high Vimentin expression compared to HepG2. Since SNU449 is an intermediate stage cell line, it acquired the mesenchymal characteristics, hence the decrease in the epithelial markers and the increase in the mesenchymal markers. As for U2OS, it showed low E-cadherin expression, high N-cadherin expression and low Vimentin expression compared to SNU449 and comparable E-cadherin and Vimentin expression and high N-cadherin expression compared to HepG2. This result can be attributed to the fact the U2OS is not classified as osteoblastic nor fibroblastic, meaning it has both epithelial and mesenchymal characteristics (Pautke et al., 2004). This indicates that U2OS can be in a later stage of EMT compared to HepG2 and an earlier stage of EMT compared to SNU449.

The protein expression of Vimentin were also assessed in HepG2, SNU449 and U2OS using western blotting (Figure 13). Vimentin protein expression was comparable in U2OS and SNU449 while it was not detected in HepG2. These results confirm what is known about the three cell lines. Two bands were observed in SNU449 while only one band was observed in U2OS. The second band observed in SNU449, could be a result of
alterative splicing or post-translational modification (Harvey & Cheng, 2016; Martin et al., 2018). As for HepG2, the protein is not detected while mRNA is expressed. This could be due to the degradation of the mRNA or inhibition of translation. Our Vimentin protein expression results of HepG2 and SNU449 match the results we obtained computationally via Expression Atlas.

4.4. Potential Targets of hsa-miR-590-3p and the FOXA2-VCAN Pathway

FOXA2 has been previously identified as a potential downstream target gene of miR-590-3p in epithelial ovarian cancer (EOC) (Salem et al., 2018). Forkhead box A2 (FOXA2) is a member of FOXA family, also known as hepatocyte nuclear factor (HNF). FOXA2 is a transcription factor involved in embryo development regulation and metabolism and homeostasis during adult stage. It has been reported that it is involved in hepatic specification and is important for hepatic glucose and lipid homeostasis (J. Wang et al., 2014). Several studies reported that FOXA2 has a dual role in cancer development; it has tumor-suppressive and tumor-promoting effects in various cancers (Salem et al., 2018; J. Wang et al., 2014). Interestingly, FOXA2 was found to be sexually dimorphic in HCC, meaning it is tumor-suppressive in females and tumor promoting in males (J. Wang et al., 2014).

A recent study suggests that VCAN is a downstream target gene of FOXA2 and that there is a negative correlation between their mRNA expression in EOC (Salem et al., 2018). Versican (VCAN) is a member of aggrecan/versican proteoglycan family and is a main component of the extracellular matrix. It has been reported to play an important role in tumor development through cell adhesion, proliferation, migration, invasion and angiogenesis (Salem et al., 2018; Sun, Gao, Gu, Cheng, & Chen, 2018).

miRanda-mirSVR was used to align the mRNA of FOXA2 against hsa-miR-590-3p to assess the binding sites of our miRNA on its mRNA (Figure 9). The mRNA expression of FOXA2 and VCAN in U2OS and HepG2 was assessed computationally using The Human Protein Atlas. FOXA2 mRNA expression is higher in HepG2 compared to U2OS
while VCAN mRNA expression is comparable in both cell lines (Figure 10). The protein expression of FOXA2 and VCAN in the HCC cell lines, HepG2 and SNU449, and in the OS cell lines, U2OS, MG-63 and Saos-2 was assessed computationally using Expression Atlas. FOXA2 protein expression is higher in HepG2 compared to SNU449 while VCAN protein expression is higher in SNU449 compared to HepG2 (Figure 11). As for the OS cell lines; FOXA2 protein expression is higher in U2OS compared to MG-63 and Saos-2 while VCAN protein expression is higher in MG-63 and Saos-2 compared to U2OS (Figure 11).

The mRNA expression of FOXA2 and VCAN were assessed in HepG2, SNU449 and U2OS via RT-PCR (Figure 15). In U2OS, a negative correlation was observed between FOXA2 and VCAN mRNA expression; FOXA2 expression is high while VCAN expression is low. This result confirms our *in silico* result obtained via The Human Protein Atlas. In HepG2 and SNU449, no statistically significant change was observed among the mRNA levels of FOXA2 and VCAN. The HepG2 result does not match our *in silico* result obtained via The Human Protein Atlas, as there is a negative correlation computationally.

### 4.5. Potential Downstream Target Genes of miR-590-3p and Cell Stemness

Another gene that has been previously reported as a target of hsa-miR-590-3p in EOC is SOX2 (Shawer, 2016). SOX2, also known as SRY (sex determining region on the Y chromosome) box 2, is a member of the Sox family and is essential to reprogram differentiated cells into induced pluripotent stem cells and maintain the cell’s self-renewal(Sun et al., 2013). SOX2 was reported to participate in oncogenesis and tumor progression of several cancers, including OS. It has also been reported that SOX2 is a predictor of poor prognosis for post hepatectomy HCC patients (Sun et al., 2013).

miRanda-mirSVR was used to align the mRNA of SOX2 against hsa-miR-590-3p to assess the binding sites of our miRNA on its mRNA (Figure 9). The mRNA expression of SOX2 in U2OS and HepG2 was assessed computationally using The Human Protein
Atlas. SOX2 mRNA expression is higher in U2OS compared to HepG2 (Figure 10). The protein expression of SOX2 in OS cell lines, U2OS, MG-63 and Saos-2 was assessed computationally using Expression Atlas. SOX2 is expressed in U2OS and is not detected in MG-63 and Saos-2 (Figure 11).

The mRNA and protein expression of SOX2 were assessed in HepG2, SNU449 and U2OS using RT-PCR and western blotting, respectively (Figure 14). U2OS and SNU449 showed comparable mRNA expression while HepG2 showed lower mRNA expression. HepG2 and U2OS mRNA results match the computational results we obtained via The Human Protein Atlas. Surprisingly, HepG2 and SNU449 showed comparable protein expression while U2OS showed low expression compared to HepG2 and SNU449. Since HepG2 is an early-stage well-differentiated cell line, we expected that SOX2 protein levels in HepG2 would be lower than SNU449 and U2OS.

4.6 Expression and Localization of hsa-miR-590-3p and SOX2

After assessing the expression of our potential downstream target genes of hsa-miR-590-3p, we wanted to assess the expression and the localization of hsa-miR-590-3p in relation to one of its downstream target genes, SOX2. This was carried out using U2OS and SNU449 cell lines. A negative correlation between the hsa-miR-590-3p expression and the SOX2 expression was observed in both cell lines. Cells that showed hsa-miR-590-3p signal showed minimal SOX2 fluorescence (red circles, Figures 16 and 17) and cells that did not show hsa-miR-590-3p signal, SOX2 fluorescence was detected (white circles, Figures 16 and 17). In U2OS, the hsa-miR-590-3p signal was low while the SOX2 fluorescence was high while in SNU449 the opposite was observed, the hsa-miR-590-3p signal was high while the SOX2 fluorescence was low (Figures 16, 17 and 18). Through these findings, we strongly suggest that SOX2 can be a direct downstream target gene of hsa-miR-590-3p in OS and HCC.
5. Conclusion and Future Recommendations

5.1. Conclusion

In conclusion, our study revels that SOX2 can be a direct downstream target gene of hsa-miR-590-3p in OS and HCC suggesting that hsa-miR-590-3p can have a direct effect on the self-renewal and self-maintenance of cancer cells. We propose that CX3CL1, E-cadherin, N-cadherin and FOXA2 show a lot of potential as downstream target genes of hsa-miR-590-3p suggesting that hsa-miR-590-3p can have an indirect effect on EMT and in turn affecting cancer progression. Nonetheless, future studies need to be conducted to further prove our work.

5.2. Future Recommendations

Future studies are needed to further understand the role of hsa-miR-590-3p and its downstream target genes in OS and HCC. We suggest the overexpression or the knockdown of hsa-miR-590-3p to access the changes in the expression of its downstream target genes. This may reveal its action, whether tumor suppressor or oncogene, in different cancer types. Also, we suggest the examination of the expression levels of hsa-miR-590-3p and the mRNA and protein levels of its potential downstream target genes in tissue specimens of OS and HCC patients.
References


http://cancerres.aacrjournals.org/content/early/2018/05/10/0008-5472.CAN-17-3014.abstract

http://doi.org/10.1038/nmeth.2019


http://doi.org/10.1681/asn.2017111200


http://doi.org/10.1212/CON.0000000000000638


http://doi.org/10.1016/j.clinbiochem.2013.01.025


