Primary gallbladder carcinoma, originating from the gallbladder epithelium, causes malignant tumors but it typically lacks clinical manifestations in the early stages. Thus, early diagnosis in patients only occurs ~19.1% of the time [1]. Most patients have progressed to middle and advanced stages of the disease when finally diagnosed. Also, cures after surgical resection are relatively rare and usually accompanied by poor prognosis. For example, five-year survival for gallbladder resection in American and European countries is only 5–13%. Recently, research suggested that abnormal molecular biological mechanisms are associated with gallbladder carcinoma and that these may be used to identify molecular markers for early diagnosis and treatment targets [2, 3].

MicroRNAs (miRNAs) are non-coding single-stranded RNA molecules involved in genetic expression and regulatory activities after transcription in plants and animals. miRNA-145 is located at the 5q32 region of the long arm of human chromosome 5, and like other miRNAs it neither contains an open reading frame nor encodes for proteins. Previous studies indicate that expression of miRNA-145 is down-regulated in almost all carcinoma tissues [4-9]. Some studies have reported that miRNA-145 expression is down-regulated in breast cancer tissue, and that miRNA-145 can inhibit propagation of breast cancer cells and induce apoptosis [10]. However, the role of miRNA-145 in gallbladder carcinoma and the underlying molecular mechanisms have not yet been elucidated.

In this study, we characterized the biological function of miRNA-145 in gallbladder carcinoma by measuring differential expression of miRNA-145 in normal gallbladder epithelial cells and GBC-SD (gallbladder series) cells using miRNA chip technology. Propagation, apoptosis, and invasion properties of each cell group were tested using MTT, a clone-formation assay, flow cytometry, Western blot, and Transwell assays. Results: Expression of miRNA145 was observed to be down-regulated and GBC-SD cell clones transiently transfected with hsa-miRNA-145 were substantially reduced compared with controls (p<0.01). We observed that GBC-SD cells transfected with hsa-miRNA-145 and double-positive (Annexin V and PI) for apoptosis were more numerous than controls. Moreover, GBC-SD cells over-expressing miRNA-145 had significantly greater expression of apoptosis-related protein, caspase-3. A Transwell assay confirmed that GBC-SD cells over-expressing miRNA-145 that migrated to the lower chamber were fewer compared with controls. Post-transcriptional regulation of gene expression was measured using dual-luciferase reporter assays and data show that miRNA-145 facilitates the inhibition of GBC-SD cell growth and invasion while inducing apoptosis by targeting DFF45. Conclusion: Thus, we speculate that miRNA-145 facilitates inhibition of GBC-SD cell growth and invasion while inducing apoptosis by targeting DFF45; however, miRNA-145 does not directly affect the GBC-SD cell cycle.

Keywords: miRNA-145; DFF-45; MTT; flow cytometry; clone formation assay
behavior for the purpose of finding molecular markers for early diagnosis. We believe that this study will provide a platform to enable future identification of potentially effective treatment targets.

2 Materials and methods

2.1 Cell culture and gallbladder carcinoma tissue

Normal gallbladder epithelial cells were extracted from healthy gallbladder tissue isolated from gallbladder polyps obtained from cholecystectomy patients. The isolated tissue was pathologically extracted from polyp sections that had no obvious inflammation.

A GBC-SD human gallbladder low-differentiation carcinoma cell line was purchased from Shanghai Institutes for Biological Sciences, CAS. The GBC-SD cell line and normal gallbladder tissue were cultured at 37°C and 5% CO2 in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) and 1% glutamine. All fresh tissue samples used in this study were isolated at the Second Xiangya Hospital, the Third Xiangya Hospital and the Hunan People’s Hospital from specimens surgically resected between January 2011 and June 2013, with tissue sizes approximating 0.8 cm × 0.8 cm × 0.6 cm. Among the 15 gallbladder carcinoma tissue specimens, six were extracted from male patients and nine were extracted from female patients. All patients were between 40 and 67 years-of-age (mean 54.3 years). Prior to surgery, no gallbladder carcinoma patient underwent radiotherapy or chemotherapy, and each patient had complete medical records. After surgery, all patients were confirmed as having gallbladder carcinoma after pathological diagnoses. Controls were composed of the same 15 patients, with control tissues being derived from adjacent tissues that were ≥ 3 cm in distance from the tumor and showing no cancerous invasion on pathological diagnosis. Less than 0.5 h after isolation, tissue specimens were stored in Cryo Tubes with liquid nitrogen freezing until experiments.

Informed consent: Informed consent has been obtained from all individuals included in this study.

Ethical approval: The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by the authors’ institutional review board or equivalent committee.

2.2 Quantitative real-time RT-PCR

Total RNA was isolated using Trizol (Invitrogen, city, state). RNA extracts were subsequently reverse-transcribed into cDNA using a PrimeScript RT Master Mix (Takara, Japan) according to the manufacturer’s instructions. Quantitative real-time PCR was performed using the SYBR Premix Ex TaqTM kit (Takara, Japan). The specific primer sequences used for quantitative real-time PCR were as follows: hsa-miRNA-145-F: 5’-AGTCGCTAGCCCACCCTGGCTGCTACAGATG-3’, and hsa-miRNA-145-R: 5’-ATCGGAATTCGGACAGCCTTCTTCTTGAACC-3’. Primers were synthesized by Shanghai RiboBio Co. Ltd. (China). Expression of miRNA-145, relative to U6, was measured using the 2−ΔΔCt method. Results were analyzed according to the manufacturer’s instructions. Experiments were performed independently, in triplicate, and data from a representative experiment are shown.

2.3 miRNA microarray and data analysis

Global miRNA expression data were obtained using Affymetrix miRNA 1.0 arrays (Affymetrix). Total RNA samples were labeled using an Affymetrix FlashTag Biotin HSR RNA Labeling Kit according to the manufacturer’s protocol. Briefly, 500 ng of each total RNA sample was poly(A)-tailed at 37°C for 15 min using poly (A) polymerase enzyme and ATP. The resultant mixture was biotinylated by ligating biotin-labeled fragments to the 3′-ends of RNA molecules. Labeled samples were hybridized on miRNA 1.0 arrays at 48°C and 60 rpm for 16 h. Arrays were subsequently washed and stained using the standard Affymetrix protocol with an Affymetrix Hybridization, Wash, and Stain Kit using a FS 450 fluidic station instrument. Arrays were scanned using an Affymetrix GeneChip Scanner 3000 instrument. An Affymetrix GeneChip Command Console 1.1 software package was used for data analysis.

2.4 Construction of mir-hsa-miRNA-145 expression vector, strains and plasmids

E. coli Top10 (Invitrogen) cells and plasmid pLL3.7 (Addgene) were used to construct eukaryotic miRNA expression vectors. First, DNA was extracted from miRNA-145 expressing cells and vector-transformed E. coli Top10 cells. The NCBI database was used to design primers that facilitated amplification of the full-length miRNA-
145 molecule. PCR amplification of the template was subsequently performed and the amplicons were digested with restriction endonucleases compatible with the vector multi-cloning site. The vector was digested with compatible restriction enzymes, and both insert and plasmid were separated using 1% agarose gel electrophoresis. Gel extraction procedures were subsequently used to purify the vector and associated insert. The hsa-miRNA-145 fragments were ligated to the plasmid backbone and the ligated products were used to transform competent E. coli cells. Resultant transformants were screened for the presence of the hsa-miRNA-145 inserts.

2.5 Transient transfection of expression plasmid, stable transfection of cells, and clone selection

Cells were subcultured in a 6-well plate and grown until they reached 60–80% confluence and then culture media was changed to resistance-free 10% FBS DMEM complete medium. One to two hours after the media was changed, Lipofectamine 2000 (Invitrogen) was used to facilitate transfection. A total of 1 μl of the expression vector (final concentration of 2 μg/μl), 100 μl of serum-free medium and 5 μl of the transfection reagent, lip2000, were incubated at room temperature for 5 min. This reaction mixture was thoroughly mixed prior to the 5 min incubation step. Opti-MEM (Invitrogen) medium containing Lipofectamine 2000 was added, drop-wise, to an Eppendorf tube containing the requisite plasmids, and the reaction was allowed to proceed for 30 min. Finally, the reaction mixture was added to cultured cells (drop-wise) and shaken to re-suspend the associated suspension. The media was changed every 5 h.

For GBC-SD cells transfected after 48 h, complete medium containing 0.5 μg/ml G418 was used to screen for transfected clones. Media was changed every other day. Resultant cell cultures were monitored for two to three weeks, and clones visible to the naked eye were grown. Clones were then isolated to generate amplification cultures.

2.6 Apoptotic

Cells were harvested and rinsed twice with PBS and samples were diluted with 150 μl of 1 × annexin-binding buffer. Next, 5 μl of FITC-labeled enhanced-annexin V and 5 μl (20 μg/ml) of propidium iodide were added into the cell suspension. Cells were subsequently incubated in the dark for 15 min at room temperature. Flow cytometry was conducted using a FAC-SCalibur instrument (FACSVers). Data were analyzed according to the manufacturer’s instructions. We repeated the experiment three times to confirm results.

2.7 Western blot

Total protein was extracted using RIPA buffer and a protease inhibitor cocktail (Pierce, Rockford, IL). Extracted protein was quantified using a BCA protein Assay Kit (Pierce) and separated by 9% SDS-PAGE. Gels were subsequently transferred onto PVDF membranes (Millipore, Bedford MA). Next, membranes were incubated with rabbit mono-clonal anti-caspase-3 antibody (1:5,000; Epitomics, Burlingame, CA) or mouse mono-clonal anti-GAPDH antibody (1:5,000, Abcam, Cambridge, MA). Membranes were subsequently incubated with secondary antibody. Signals were measured using ECL.

2.8 Dual-Luciferase reporter assay

We plated the GBC-SD cells in 24-well plates the day before transfection. Wild-type or seed-region-mutated DFF-45 3′-untranslated region (UTR) sequences were inserted into pGL4 plasmids following double restriction enzyme digestion. Reporter plasmids were co-transfected with miRNA-145 or the control into GBC-SD cells using Lipofectamine 2000 (Invitrogen). Cells were harvested 48 h later, and relative luciferase activities were measured using a Dual-Luciferase Assay System (Promega, Madison, WI).

3 Results

3.1 Expression of miRNA-145 was down-regulated in GBC-SD cells and gallbladder carcinoma tissue

Micro-array hybridization of RNA specimens and scanning analyses showed dot-shaped florescent signals and data indicate that in GBC-SD cells miRNA-145 was down-regulated compared to normal gallbladder epithelial cells. Figure 1 shows miRNA-145 expression in gallbladder carcinoma tissues was significantly decreased compared with adjacent gallbladder tissues. These data agree with GBC-SD cell chip assay results.
3.2 Successful construction of hsa-miRNA-145 over-expression vector and hsa-miRNA-145 over-expression gallbladder carcinoma stable cell line

We constructed an hsa-miRNA-145 over-expressing vector and after comparing DNA sequencing results with the original sequence from the NCBI reference genome, we observed that the GV143-hsa-miRNA-145 sequence was identical to the original sequence and had no single base mutations.

We subsequently generated GBC-SD cell lines that stably over-expressed hsa-miRNA-145. After G418 resistance screening, we obtained GBC-SD cell lines containing stably transfected empty plasmids and GBC-SD cell lines stably over-expressing the hsa-miRNA-145 plasmid. Total RNA was extracted from transfected and untransfected cells lines prior to real-time PCR analysis (Fig. 2). Data show that hsa-miRNA-145 in cells stably over-expressing hsa-miRNA-145 was significantly elevated compared with the normal control group (untransfected GBC-SD cell group) and the empty plasmid-transfected group (p < 0.05). Compared with the empty plasmid group, the normal control group had no significant changes in hsa-miRNA-145 expression (p > 0.05).

3.3 GBC-SD cell lines stably transfected with hsa-miRNA-145 exhibited reduced growth

We studied three groups at different time points with an MTT assay, and plotted the GBC-SD growth curve (Fig. 3). Data show that on the fourth day of culture, GBC-SD cells stably transfected with hsa-miRNA-145 were inhibited 30% (p < 0.05), compared with the normal control group and empty plasmid group.

3.4 Over-expression of hsa-miRNA-145 has no significant effect on the cell cycle

Flow cytometry data for GBC-SD cells transfected with hsa-miRNA-145 and normal control group cells appear in Fig 4. Cells over-expressing hsa-miRNA-145 in the S phase were slightly increased compared with the normal control group and the empty plasmid-transfected group; however, the difference was not significant (p > 0.05).
3.5 Hsa-miRNA-145 induces apoptosis of GBC-SD cells

Flow cytometry data showed that GBC-SD cells transfected with hsa-miRNA-145 were double-positive (Annexin V and PI) and more apoptotic than the normal control and this difference was significant ($p < 0.05$; Fig. 5).

We measured expression of caspase-3 with Western blot and (Fig. 6) show that relative expression of the caspase-3 protein in GBC-SD cells transfected with hsa-miRNA-145 was higher than in normal control and the empty plasmid transfection group. These differences were significant ($p < 0.01$) and show that caspase-3 expression was up-regulated after hsa-miRNA-145 over-expression.

3.6 Hsa-miRNA-145 inhibits GBC-SD cell clone formation

Clone formation assays showed that clones formed from GBC-SD cells transfected with hsa-miRNA-145 were fewer than for controls ($p<0.01$; Fig. 7). However, compared with the empty plasmid group, GBC-SD cells not transfected were not different with respect to clones formed ($p < 0.05$).

3.7 Over-expressed miRNA-145 down-regulates expression of DFF45 mRNA and protein

Sequence analysis showed that the DFF45 3'-UTR contains a putative binding site (854 bp–876 bp, Fig. 9A) for miR-145. After transfection of GC-SD cells with miRNA-145, we measured DFF45 mRNA and protein expression. Real-time PCR and Western blot revealed that compared with controls and transfected empty plasmids, over-expressed miRNA-145 cell lines had less DFF45 transcription ($p < 0.05$) and protein expression ($p < 0.05$; Fig. 8).

We next assayed relevant reporter plasmids and miRNA for fluorescent activity and observed that cells transfected with NC and DFF45-85A, cells transfected with hsa-miRNA-145 and DFF45-854 had less fluorescent activity; the associated difference was significant ($P = 0.005281$; Fig. 9B). Compared with cells transfected with NC and DFF45-854 mutation, cells transfected with hsa-miRNA-145 and the DFF45-854 mutation had less
Figure 5. Flow cytometry indicating apoptosis of GBC-SD cells (Annexin V-FITC). A: represents normal non-transfected GBC-SD cells; B: represents hsa-miRNA-145 transfection group.

Figure 6. Expression of caspase-3 protein in GBC-SD cells according to Western blot.

Figure 7. Hsa-miRNA-145 inhibits GBC-SD clone formation. A: cells over-expressing hsa-miRNA-145; B: GBC-SD cells transfected with empty plasmids; C: normal non-transfected GBC-SD cells.
fluorescent activity; however, the difference was not significant ($P = 0.4784$; Fig. 9C). With EZH2, a known target gene of miR-101 as a positive control, we observed that compared with cells transfected with NC and EZH2-CDS854, cells transfected with hsa-miR-101 and EZH2-CDS854 had less fluorescent activity. Thus, DFF45 is a direct target for miRNA-145 in gallbladder cancer cells.

4 Discussion

The pathogenesis and development of tumors predominantly originate from homeostatic regulation disorders pertaining to particular molecular events, including activation of proto-oncogenes and inactivation of tumor suppressor genes [11]. Tumor occurrence subsequently results in changes in inherent cell behavior, which are reflected in tumor cell propagation, evasion from apoptosis and immunological surveillance, epithelial to mesenchymal transition (EMT), migration, and clone formation [12]. Effective tumor suppression changes propagation, apoptosis, and migration. After tumor treatment, not all tumor cells are eliminated; but it is hoped that their growth will be suppressed to promote treatment and patient survival.

As a tumor suppressing miRNA, miRNA-145 recently gained attention due to its role in tumorigenesis and tumor development. Importantly, miRNA-145 has been observed to exhibit low expression in many diverse cancers [13-18]. Thus, miRNA-145 plays an important regulatory role in tumor cell genesis, propagation, apoptosis, EMT, and migration.

Our previous studies showed that expression of miRNA-145 in GBC-SD cells and gallbladder carcinoma tissue were down-regulated, suggesting that miRNA-145 may affect the genesis and development of gallbladder carcinoma by regulating the biological function of gallbladder cells. This is consistent with the previous report [19]. An MTT assay showed that on the fourth day of cell culture, GBC-SD cells with high hsa-miRNA-145 expression had 30% reduction in growth, compared with normal gallbladder cells or cells transfected with empty plasmids. However, compared with normal GBC-SD cells, cells transfected with empty plasmids were not changed with respect to cell number. Therefore, decreased number of GBC-SD cells is due to hsa-miRNA-145 over-expression. Several studies have shown that miRNA-145 indirectly regulates the cell cycle by directly targeting cyclin proteins or by targeting oncogenes such as c-myc. However, it has also been suggested that miRNA-145 may target relevant members of the IGF signal pathway to inhibit cell propagation. In colon cancer, insulin receptor substrate-1 (IRS-1) binds to type I insulin-like growth factor receptor (IGF-IR). The resultant complex is a strong mitogen that conveys the signals of mitosis, anti-apoptosis and cell differential inhibition and regulates cell growth and propagation, causing cell mutations and tumor genesis [20].

Slaby’s group [21] reported that both the 3′-untranslated region (3′UTR) of IRS-1 and IGF-IR3′UTR were directly regulated by miRNA-145. It is therefore...
feasible that miRNA-145 affects GBC-SD cell numbers by inhibiting the propagation of gallbladder carcinoma cells. However, we observed that miRNA-145 in GBC-SD cells had no obvious effect on the cell cycle, suggesting that miRNA-145 does not suppress cell numbers by hindering the cell cycle. This may be due to heterogeneity and complexity of tumorigenesis. Cancerous processes associated with normal tissue after pathological stimulation are likely to activate different genetic combinations. miRNA-145 is likely to target hundreds of associated genes while mediating pathological genesis and development. Likewise, it is likely that miRNA-145 expression may also be regulated by hundreds of genes. This suggests that miRNA-145 may have different effects on the cell cycle in cancerous processes in different tissues.

Thus, the mechanism for miRNA-145 inhibition of GBC-SD cell growth is of interest. Apart from decreased propagation, decreased cell numbers may result from increased apoptosis. In this study, we observed that over-expression of miRNA-145 in GBC-SD cells decreased DFF45 transcription and expression, suggesting that miRNA-145 can regulate DFF45 in gallbladder cancer cells. This is in line with previous research that shows DFF45 has been reported as one of the targets of miRNA-145 in colon cancer [22].

DNA fragmentation factor (DFF), also known as CAD (caspase-activated DNase) [23, 24], is one of the direct substrates of Caspase3, including a basic protein DFF40 with molecular weight of 40 kDa, and an acidic protein of 45 kDa. Among them, DFF40 is a catalytic subunit of DFF, while DFF45 is the regulation subunit of DFF. DFF40 acts as ribozyme of DNA [25, 26], when DFF40 is activated, DNA can be cut into 180-200 bp pieces, representing specific molecular characterization as DNA Ladder showed in apoptosis, and the activated DFF45 has an inhibitory effect on DFF40. Studies showed that in human, mouse, rat, zebrafish, drosophila and chicken and other organisms, DFF40 and DFF45 are conservative in structure and function, and are expressed in many human tissues. It has been indicated that DFF40 and DFF45 have wide-spectrum characteristics in the regulation of apoptosis [27].

During the regulation of apoptosis, DFF45 acts as a molecular chaperone of DFF40. Having finished the protein translation of DFF40, DFF45 can bind to the DFF40 nascent chain to help it fold correctly, and in the meantime it enters the nucleus as a protein complex. When the external apoptosis signals stimulate and induce the response of apoptosis, the activated Caspase3 can cut the two sites in DFF45: the Asp117 and Asp224, thus resulting in the action zone damage between DFF45 and DFF40, and the release of DFF40 occurs successively. DFF40 forms catalytic activated homo oligomers, which degrade chromosome DNA, and afterwards produce apoptotic bodies, causing cell apoptosis [28-29].

One of the important characters of cancer cells is that they can escape apoptosis, which may be due to the dysregulation of apoptosis within tumor cells. It has been found that DFF45 has a certain role during the occurrence and development of cancer. Examination of normal endometrial tissue from 20 cases of hysterectomy due to uterine fibroids found that SiSo cell receptor binding cancer antigen (receptor binding cancer antigen-expressed on SiSo cells – SiSo, RCAS1 and DFF45) shows consistent negative correlation to DFF45 at different stages, which demonstrates that DFF45 may be involved in apoptosis resistance of endometrial tumors [28]. In the NB-1 and NB-C201 cell lines of human neuroblastoma, there is a homozygous deletion in the chromosome region 1p36.2 to p36.3, which includes DFF45, suggesting that DFF45 may play a key role in the occurrence of human neuroblastoma [29]. In addition, a relationship between the differential expression of DFF45 and the incidence of cervical cancer, colorectal cancer and lung cancer has been also reported. Given the role of DFF45 in the development of various types of cancer, is further study of DFF45 in gallbladder carcinoma is warranted. Protein assays showed that over-expression of miRNA-145 could decrease expression of DFF45, indicating that miRNA-145 can suppress DFF45 translation. Using a fluorescent reporting system for verification, we observed that a region (854 bp–876 bp) in the DFF45 mRNA molecule was suppressed by miRNA-145. This suggests that the 854 bp–876 bp region is essential for miRNA-145 to suppress DFF45 mRNA translation. The result also shows that miRNA-145 targets the 854 bp–876 bp section of the DFF45 mRNA molecule to down-regulate DFF45 expression in gallbladder cancer cells. These results are similar to those previously reported regarding miRNA-145 in intestinal cancer. According to a previous study, miRNA-145 can down-regulate DFF45 expression to prevent colon cancer by targeting the 854 bp–876 bp mRNA section [30]. In accordance with our previous findings, over-expression of miRNA-145 in GBC-SD cells increases apoptosis and significantly up-regulates caspase-3. This suggests that in gallbladder cancer cells, miRNA-145 might up-regulate caspase-3 resulting in release of DFF40 while down-regulating DFF45 expression by targeting the 854 bp–876 bp mRNA section. These results suggest that miRNA-145 disables DFF40 suppression by DFF45 thereby facilitating caspase-3-induced apoptosis. Thus, miR-145 can increase apoptosis of gallbladder cancer cells by directly suppressing DFF45, suggesting that miR-145 may be a novel treatment target for gallbladder cancer.
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References


