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Original Articles

miR-145, targeting high-mobility group A2, is a powerful predictor of patient outcome in ovarian carcinoma

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ABSTRACT

MicroRNA-145 (miR-145) expression is downregulated in several human cancers, but its clinical and functional relevance to ovarian carcinoma has not yet been elucidated. This study addressed the hypothesis that miR-145 serves as a prognostic biomarker and a tumor suppressor that regulates the expression of high-mobility group A2 (HMGA2) oncoprotein in ovarian cancer.

Here, we found that low miR-145 expression and HMGA2 overexpression determined by qRT-PCR and immunohistochemistry significantly correlated with advanced stage, lymph node involvement, and distant metastasis in 74 ovarian carcinomas. Low miR-145 expression significantly correlated with tumor recurrence and worse overall survival (HR=8.62, $P = 0.039$). Transfection of pre-miR-145 resulted in reduced cell growth and migration, and increased apoptosis of ovarian cancer cells by TUNEL, colony forming, and cell migration assays. MiR-145 was found to directly target HMGA2 by luciferase assay and Western blotting.

Our findings suggest that miR-145 functions as a tumor suppressor in ovarian cancer and directly targets HMGA2 oncoprotein. Low miR-145 and high HMGA2 expressions are potential biomarkers of poor prognosis of ovarian carcinoma and miR-145 is the more powerful predictor of patient outcome.

Introduction

Ovarian carcinoma is a highly lethal gynecologic cancer due to frequent recurrence and metastasis. Despite its high incidence and mortality rate, the mechanisms involved in the prognosis and recurrence of ovarian carcinomas remain unclear. Recent evidences suggest that altered microRNA (miRNA) levels are related to the oncogenesis of many human cancers [1,2], including ovarian carcinoma [3,4]; hence, miRNAs could be used to formulate a prognosis and may be potential targets for cancer therapy [5,6]. Understanding the functions and expression levels of miRNAs may provide insights into the pathobiology of ovarian carcinoma on a molecular level and may reveal new prognostic biomarker and therapeutic targets.

MiRNAs are small non-coding RNAs that inhibit the expression of multiple genes at the post-transcriptional level via partial base pairing to the 3′ untranslated region (UTR) of their targets. MiRNAs are involved in the regulation of various cellular processes, such as the cell cycle [7] and apoptosis [8]. Furthermore, aberrant expression of miRNAs promotes tumorigenesis, metastasis and other features of cancer [9]. Gene expression studies show that hundreds of miRNAs are dysregulated in cancer cells and functional studies reveal that many miRNAs are involved in the control of oncogenes or tumor suppressors [1]. Recent studies demonstrated that aberrant miRNA expression is correlated with the histological subtype and prognosis of human cancer, including ovarian cancer [4].

MiR-145 was initially reported to be reduced expression in colorectal neoplasia [10]. Subsequently, downregulation of miR-145 has been demonstrated in human lung, colon and bladder cancers [11]. MiR-145 acts as a tumor suppressor that inhibits the growth, invasion and migration of colon cancer cells, both *in vitro* and *in vivo*, by specifically silencing c-Myc or insulin receptor substrate 1 [12]. A high-throughput microarray study also revealed differential expression of miR-145 in ovarian carcinomas [3], however, its clinicopathological impact on ovarian carcinomas has not yet been reported. We, therefore, in this study, investigated the differential expression of miR-145 and its clinicopathological implication in ovarian carcinomas focused on high grade ovarian serous

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carcinomas (HG-OSC), which is the most common and lethal type, accounting for 80% of ovarian carcinoma. We further evaluated whether miR-145 functions as a tumor suppressor in ovarian cancer cells, and whether miR-145 directly targets the *HMGA2* oncogene, which is overexpressed in multiple malignant neoplasms including ovarian cancer [13–15], and which is a potential target of miR-145 by the TargetScan and miRNA.org software programs.

HMGA2 regulates the transcription of several genes by enhancing or suppressing transcription factor access; hence, it is involved in many fundamental cellular processes, including cell-cycle control, differentiation, and cellular senescence [16,17]. HMGA2 has emerged as a candidate biomarker because it is overexpressed in many human cancers and is undetectable in the majority of normal adult human tissues. In ovarian carcinomas, high HMGA2 immunoreactivity is associated with advanced stage disease and tumor aggressiveness [18,19]. HMGA2 expression is reportedly regulated at the post-transcriptional level by let-7 miRNA [20], which is downregulated in many solid organ cancers [21], including ovarian cancer [22]; however, to our knowledge, the interaction between miR-145 and HMGA2 in human cancers has not yet been investigated.

Herein, the expression levels of miR-145 and HMGA2 in HG-OSC tissue samples were examined and correlated with the clinicopathological parameters. The function of miR-145 in the growth and apoptosis of ovarian carcinoma cells was also examined, and targeting of the *HMGA2* gene by miR-145 was investigated by luciferase assay and Western blotting.

Materials and methods

Patients and tissue samples

A total of 74 HG-OSC tissue samples, including 35 fresh samples and 39 formalin-fixed paraffin embedded (FFPE) samples, were used in the study. The fresh tissue samples were obtained from ovarian cancer patients who underwent surgery at the CHA Bundang Medical Center (CHA University, Korea) between 2003 and 2009. These samples were immediately frozen in liquid nitrogen and stored at –80 °C until use. The FFPE samples were collected from the archives of CHA Bundang Medical Center and originated from tissues collected between 1998 and 2011. Clinical and pathological data were retrieved from clinical databases and pathology report archives. Clinical staging was evaluated according to the criteria of the International Federation of Gynecology and Obstetrics (FIGO) staging system [23]. As a control, 10 normal fallopian tubes, which are considered as an origin of HG-OSCs in either familial or sporadic ovarian cancers [24], were collected from patients who underwent hysterectomies for leiomyomas. This study was approved by the Ethical Committee of the CHA Bundang Medical Center and informed consent was obtained from each patient prior to surgery.

The ages of the patients enrolled in the study ranged from 33 to 83 years (median age, 58 years) and 41 patients (55.4%) were aged ≥ 55 years. Of the 74 HG-OSC patients enrolled, 21 (28.4%) had early clinical stage disease (I/II) and 53 (71.6%) had advanced clinical stage (III/IV) disease. Lymph node involvement and distant metastasis were detected in 38 (51.4%) and 19 (25.7%) cases, respectively. The patients were treated with a first-line chemotherapeutic regimen consisting of paclitaxel and cisplatin or carboplatin after radical surgery. The mean follow-up interval was 40.13 months (range, 2–139 months). Among the 74 patients included in the study, 53 survived without disease and 21 died of ovarian carcinoma.

Cells, media and culture conditions

A human ovarian surface epithelial cell line (HOSEpiC) and three human ovarian cancer cell lines (SKOV3, OVCAR3 and A2780) were purchased from the American Type Culture Collection (Rockville, MD, USA) and the European Collection of Cell Cultures (Wiltshire, UK). A2780 and OVCAR3 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 mg/ml streptomycin. SKOV3 cells were cultured in McCoy's 5A medium supplemented with 10% FBS, 100 units/ml penicillin and 100 mg/ml streptomycin. The cell lines were incubated at 37 °C in a humidified atmosphere consisting of 5% CO_2 and 90% humidity.

Detection of miR-145 by quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from fresh tissues and the HOSEpiC, SKOV3, OVCAR3, and A2780 cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The miRNeasy FFPE Kit (Qiagen, Hilden, Germany) was used to extract RNA from FFPE tissues, according to the manufacturer's protocol. There are increasing evidences that miRNAs show enhanced stability in both plasma [25] and FFPE [26] whereas RNA degradation usually occurs during tissue processing and long-tem storage. The possible reason why miRNA is more stable than mRNA in FFPE is either the lack of structure, lack of a specific nucleotide target sequence, or their small size [27]. Therefore, miRNAs can be stable in FFPE blocks over a decade, which makes archival FFPE tissues suitable for miRNA analysis. To generate cDNA, total RNA samples were reverse transcribed using specific miRNA primers and reagents from the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The threshold cycle, which is defined as the cycle number at which the fluorescence exceeds a fixed threshold, was determined using default threshold settings. All PCR reactions were performed in triplicate and gene expression relative to that of *RNU48* was calculated using the $2^{-\Delta\Delta Ct}$ method.

Transfection of cells with pre-miR-145

A chemically modified miR-145 precursor (pre-miR-145; 5′-GUCCAGUUUUCCCAGGAAUCCCU-3′) was purchased from Ambion (Applied Biosystems). To transfect cells, 45 pmol of pre-miR-145 was diluted in 500 μl of serum-free McCoy's 5A media and 5 μl of Lipofectamine 2000 reagent (Invitrogen). The transfection mixture was added to 7×10^5 cells in a 60 mm dish containing 4 ml of McCoy's 5A medium supplemented with 10% FBS. The cells were harvested 24, 48 and 72 h after transfection and prepared for the subsequent study.

TUNEL assay

To determine whether overexpression of miR-145 promotes tumor cell death, TUNEL assays of SKOV3, OVCAR3 and A2780 cells transfected with pre-miR-145 were performed. After transfection, apoptotic cells were identified using the *In Situ* Cell Death Detection Kit (Roche, Mannheim, Germany). The cells (1×10^6) were fixed with 75% ethanol for 2 h at –20 °C, washed twice with PBS, and then incubated with 0.1% Triton X-100 and 0.1% sodium citrate for 2 min on ice. After two additional washes with PBS, the cells were incubated with the TUNEL labeling mixture for 1 h at 37 °C in the dark. The samples were then washed twice with PBS and analyzed by fluorescence-activated cell sorting (Becton Dickinson, Franklin Lakes, NJ, USA).

Wound healing assay

Cells were seeded into 24-well tissue culture plates and grown to confluency. An acellular area was created by scraping the cell surface using a sterile pipette tip. The wounded monolayers were washed twice with PBS to remove floating cell debris. The monolayers were then incubated in cell culture medium and the rate of defect closure was monitored for 16 h. Individual cells were quantified as an average of at least five fields for each experiment.

Colony forming assay

Cells were seeded into 6-well plates at a density of at 1×10^5 cells per well. The following day, the cells were transfected with pre-miR-145 and incubated for 48 h. The transfected cells were then replated into 6-well culture dishes at a density of 300 cells per well. After 14 days, the colonies were fixed with 4% paraformaldehyde for 10 min, visualized using hematoxylin and then counted. Groups containing greater than 50 cells were scored as colonies.

Luciferase reporter assay

The putative miR-145 binding site within the 3′-UTR of *HMGA2* (NCBI transcript NM_003483) was identified using the TargetScan algorithm (targetscan.org). Oligonucleotides containing the wild-type (WT) or mutant (MT) miR-145 binding site were cloned into the pGL3-control vector (Ambion, Austin, TX, USA) at the NheI and XhoI sites. For luciferase assays, 1×10^5 cells were seeded into 24-well plates 1 day before transfection. OVCAR3 cells were co-transfected with 100 ng of pGL3 containing the WT or MT 3′-UTR of *HMGA2*, 5 ng of pRL-TK (Promega, Madison, WI, USA), and 50 pmol of pre-miR-145 or mirVana™ negative control miRNA (Applied Biosystems), using Lipofectamine 2000 reagent. Luciferase activity was measured 48 h after transfection using the Infinite 200 PRO plate reader (Tecan, Vienna, Austria). All experiments were performed in triplicate and data were normalized to Renilla luciferase activity.

Western blotting

To determine whether overexpression of pre-miR-145 suppressed HMGA2 protein expression in SKOV3, OVCAR3 and A2780 cells, western blotting was performed as described previously [28]. The blots were incubated with an anti-HMGA2 monoclonal antibody (1:1000 dilution; Novus Biologicals) or an anti-β-actin mouse monoclonal antibody as a control (1:2000; Sigma, St. Louis, MO, USA). Antibody de-