Original research

TARGETING THE MUTANT REGION IN *K-RAS* RNA INTERFERENCE CONTROLLING CELL PROLIFERATION AND PROGRAMMED CELL DEATH IN LIVER CANCER CELLS

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Abstract: **Background** Hepatocellular carcinoma (HCC) is a high-incidence and significant cause of cancer globally. HCC is the world's third most common death-related cancer and the sixth most common tumor. In HCC, various cellular signaling is activated to ensure malignancy transformation, angiogenesis, and metastasis. The most efficient signaling pathway in cancer is mitogen-activated protein kinase (MAPK), which controls malignancy and regulates apoptosis. The mutant *k-ras* gene led to the activation of the RAS protein with GTPase activity, which stimulated hepatocellular proliferation and transformation. Here we aim to investigate the correlation between the knock-down of *k-ras* and HCC evaluation *in-vitro*.
Material and Methods We used the HepG2 cell line to investigate the direct connection between the *k-ras* expression profile and RAF/MEK pathway using a respective siRNA antagonist *k-ras*. **Results** Interestingly, the siRNA antagonist *k-ras* altered cell morphology and a number of the living HepG2 cells. In addition, the transfection of HepG2 cells with the firstly designed siRNA successfully reduced the expression profile *k-ras* and the relative gene expression of Raf-1 and Mek1, the downstream targets of RAS signaling. Furthermore, interleukin 8 (IL-8) and interleukin 6 (IL-6) were monitored in the fluids media at different time points following transfection. Both IL-6 and Il-8 production significantly increased in cells transfected with siRNA targeting *k-ras*.
Conclusion Our findings provide evidence for the influential role of the *k-ras* mutated gene in HCC development and suggest the possible regulation of this gene as a potential treatment for HCC.

Keywords:RNA interference; Hepatocellular carcinoma; *k-ras* gene; Cell proliferation

INTRODUCTION Hepatocellular carcinoma (HCC) or hepatoma is the most cancer in liver tissue, considered the sixth most common malignancy and the second leading cause of death globally [1]. It is a highly feared form of cancer, often detected in the advanced stages, with limited treatment [2]. Hepatocarcinogenesis is caused by a multistep process involving different genetic modifications that lead to the pathogenesis of HCC [3]. A malignant cell phenotype starts with mutant hepatocytes and then produces mitogens, which activate cellular receptors and intracellular signaling pathways [4]. Carcinogenic cells activate upon DNA damage that causes several changes and mutations of cellular genes in response to several risk factors like aflatoxins, alcoholism, intoxication, and obesity with diabetes. Importantly, co-infection with HBV and HCV is responsible for approximately 80% to 90% of all cases of HCC worldwide [5]. HCC is a primary liver malignancy that occurs predominantly in a patient with acute, chronic liver diseases and cirrhosis; however, up to 25% of patients have no history of cirrhosis or risk factor for it [6]. The principal risk factors of HCC are HB/C viruses, alcohol, and chronic inflammation. This risk factor stimulates hepatic fibrosis and cirrhosis, ultimately causing HCC [7].

Several signaling pathways contribute to the development and cellular homeostasis. In cancer, the development

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component of these pathways often becomes mutated or overexpressed, causing dysregulation of cellular signaling. For instance, infection with influenza A virus, HBV, and HCV play a vital role in stimulating the RAS/RAF/MAPK signaling pathway in HCC, which includes activation of the RAS oncoprotein pathway [8–10]. RAS protein is involved in signaling pathways that facilitate gene transcription, which is required for cell growth and differentiation. Over-expression or mutation in the *Ras* gene can lead to uncontrolled cell growth and cancer development [11]. Alteration of the *Ras* gene has been found in many types of cancer, including pancreatic, lung, thyroid, bladder, liver, and ovarian [12]. Noteworthy, *k-ras* is a unique *Ras* gene mutation that frequently occurs in human cancer and is a major downstream target of the epidermal growth factor (EGFR) pathway [13]. Activation of *k-ras* stimulates *b-raf* and Mek, which activate genes involved in cell proliferation via the Erk transcription factor [14]. *K-ras* and *b-raf* mutations are crucial mediators in the EGRF signaling pathway and development in human liver cancer, especially the RAS/RAF/MEK signaling pathways that play a central role in hepatocarcinogenesis [15]. Genetic alternation in the *k-ras* often leads to abnormal RAS/RAF/MEK pathway activation in HCC [16]. RNA interference (RNAi) therapy has become promising application therapy for many diseases [17]. In this way, the current study provides evidence for a sufficient effect of targeting *the k-ras* mutant gene using specific siRNA in regulating cell proliferation and stimulating programmed cell death in liver cancer cells.

MATERIALS AND METHOD

Cell lines

All cell lines were obtained from (VACSERA, Giza, Egypt) and regularly checked for mycoplasma contamination. The hepatocellular carcinoma, HepG2 cell line, was cultured in Roswell Park Memorial Institute (RPMI) 1640 media, containing 25 mM HEPS, four mM L-glutamine, and 10% of heat-inactivated bovine serum albumin (BSA) and incubated at 37°C and relative humidity of 95% [18].

Transfection protocol

HepG2 cells were maintained in a complete RPMI medium and were overnight cultured in 6-well plates with confluency of 70-80%. HepG2 cells were then transfected with a respective siRNA antagonist *k-ras* (5`-AAUUAGCUGUAUCGUCAAGGCACUCUU-3`) or anti-Luciferase (5`-AACUUACGCUGAGUACUUCGA-3`) which served as control (Khalil et al., 2016). According to the manufacturer's protocol, the cells were transfected with 500 ng of siRNA/well using 20µl HyperFect transfection kits (Qiagen, USA) prepared in 500 µl optimum media. Transfected cells were incubated for two days. The knockdown efficiency of *KRAS* expression and relative expression of *RAF1* and *MEK1* genes was assessed using qRT-PCR [19].

Proliferation and cytotoxic assay

For proliferation assay of transfected HepG2 cells with anti-*KRAS*, cells were cultured in duplicate in a 6-well plate at 10X104 cells per well. Cell morphology was monitored using the inverted microscope. The number of living cells upon transfection was counted by using a hemocytometer. In brief, the old media was discarded, and the cells were washed twice with phosphate buffer saline (PBS) before trypsinizing by 3 min incubation at 37ºC. Then a suitable volume of complete RPMI media was added to the trypsinized cells, and the number of cells was manually counted [20, 21].

Detection of relative gene expression

To detect the relative gene expression, qRT-PCR was used to perform cDNA construction and amplification in one step using the purified total RNA as a template. Total RNA from transfected HepG2 cells was extracted 48 hrs post-transfection using TRIzol and purified using the RNeasy Mini Kit (Qiagen, USA). The relative expression of *Ras, Raf-1,* and *Mek1* in transfected HepG2 cells was detected using the QuantiTect SYBR Green PCR Kit (Qiagen, USA). The oligonucleotides in Table 1 have been used as specific primers for indicated genes. Level of amplified GAPDH was used for normalization. The following reagents were prepared for each reaction; 10µl SYBR green, 0.2 µl RNase inhibitor (20 U/µl), 0.25µl reverse transcriptase (50 U/µl), 1µl purified total RNA (100 ng/µl) and 0.5 µl from each primer up to a final volume of 20 µl using RNase free water. According to the manufacturer's protocol, the following PCR parameters were sued to construct and amplify cDNA, in one step, from a total RNA template: 50°C for 30 min, 95°C for 3 min, 40 cycles (95°C for 30 sec, 60°C for 15 sec, 72°C for 30 sec) and 72°C for 10 min.

ELISA assay

For quantitative measurement of individual interleukin, sandwich enzyme-linked immunosorbent assay (ELISA) was used to determine the concentrations of IL-6 and IL-8 using human ELISA kits (Abcam, 178013) and (Abcam, 46032), respectively. Accordingly, as described previously, HepG2 cells were seeded in a 96-well plate and transfected with siRNA antagonist *k-ras* or luciferase. Then the transfected cells were incubated for different time points, including 6, 12, 24, and 48 hrs post-transfection. Standards and samples were transferred into a new 96-well plate, and the produced interleukin was bound to the wells by the immobilized antibody. After washing, a biotinylated

interleukin antibody was added, followed by an HRP-conjugated streptavidin antibody. Finally, a TMB substrate solution was added to the wells, and color developed according to the amount of interleukin bound. The intensity of the color was measured at 450 nm [21, 22].

gene, which served as a control. The result showed significant alternation in HepG2 cell morphology compared to anti-luciferase siRNA-transfected cells and control-transected cells (Figure 1A). In addition, as presented in Figure 1B, the transfection with siRNA that

Description	Primer sequences (5'-3)
KRAS-sense	ATACAGCTAATTCAGAATCATTT
KRAS-anti-sense	CTATAATGGTGAATATCTTCAAA
RAF1-sense	TTTCCTGGATCATGTTCCCCT
RAF1-anti-sense	ACTTTGGTGCTACAGTGCTCA
MEK1sense	GACCTGCGTGCTAGAACCTC
MEK1-anti-sense	TCTGGACGCTTGTAGCAGAG
GAPDH-sense	TGGCATTGTGGAAGGGCTCA
GAPDH-anti-sense	TGGATGCAGGGATGATGTTCT

Table 1. Oligonucleotides sequence used for detection of steady state mRNA of indicated genes.

Data analysis and prediction tools

The Freiburg RNA online tool, IntaRNA program, was used to predict the possible interactions between designed siRNA and *k-ras* gene sequence. Since IntaRNA uses a heuristic to enable the low runtime, the provided energies are (close) upper bounds. Only interaction energies below or equal to 0 are visualized, and missing data (subsequences without predicted interactions) are also depicted with an energy 0. The *k-ras* sequence was obtained from the National Library of Medicine (https://www.ncbi.nlm.nih.gov). For quantification of the cycle threshold (Ct) of each investigated gene expression, delta-delta-Ct equations were used as previously described: (1) delta-Ct = Ct value for gene - Ct value for GAPDH, (2) (delta–delta-Ct) = delta-Ct for experimental–delta-Ct for control, (3) relative expression of targeted gene = (2-delta-delta ct) [9, 23]. Statistical analysis was done using the student's t-test between two groups. *P*-value ≤ 0.05 was considered statistically significant.

Results

siRNA antagonist k-ras induces alteration in cell morphology and number of living HepG2 cells. To investigate the cytotoxic effect of siRNA that antagonist k-ras, HepG2 cells were cultured in a 6-wall plate with a density of 10X104 cells/well and were incubated for 24 hours. The cells were then transfected for 48 hours with specific siRNA antagonist k-*ras* or targeting luciferase antagonist k-ras significantly reduced the number of living HepG2 cells after the incubation schedule compared with the other control transfection. This result indicates that the transfection with siRNA antagonist k-ras induces program cell death and changes cell morphology in transfected HepG2 cells.

Knockdown of KRAS reduces relative genes expression of related factors.

The knockdown efficiency of *k-ras* by indicated siRNA was assessed using qRT-PCR and specific primers for *Ras* gene expression upon transfection. Downstream targets of *Ras*, the relative expression of *Raf-1* and *Mek1* gene expression was detected by qRT-PCR. The relative expression of *the Ras* gene was significantly down-regulated by more than 80% in cells transfected with siRNA antagonist *k-ras* compared to another transfected cell (Figure 2A). Likewise, the relative expression of *Raf-1* significantly downregulated in cells transfected with siRNA antagonist *k-ras* affected by the depletion of *Ras* gene expression (Figure 2B). Moreover, the relative expression of Mek1 markedly downregulated in cells transfected with siRNA antagonist *k-ras* compared with other transfected cells (Figure 2C). This result indicates that targeting *k-ras* in HepG2 cells can reduce the expression profile of *Raf-1* and *Mek1* gene expression in the transfected cells and may regulate the RAS/RAF/MEK pathway in cancer cells.

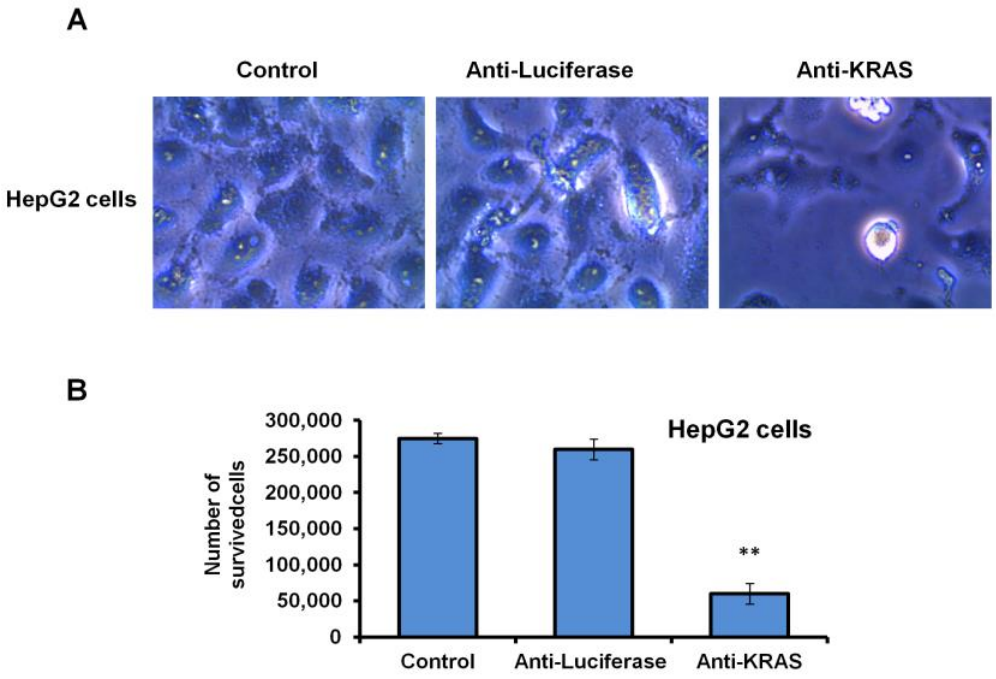

Figure 1. Cytotoxic potential of transfection protocol. (A) HepG2 cell morphology indicated by inverted microscope upon 48 hrs of transfection with the siRNA antagonist *k-ras* compared with control-transfected and untreated cells. (B) After transfection, the number of living HepG2 cells. Error panels indicate the standard deviation (STD) of three independent experiments. Student two-tailed *t*-test used for statistical analysis**,** (**) indicates *P*≤0.01.

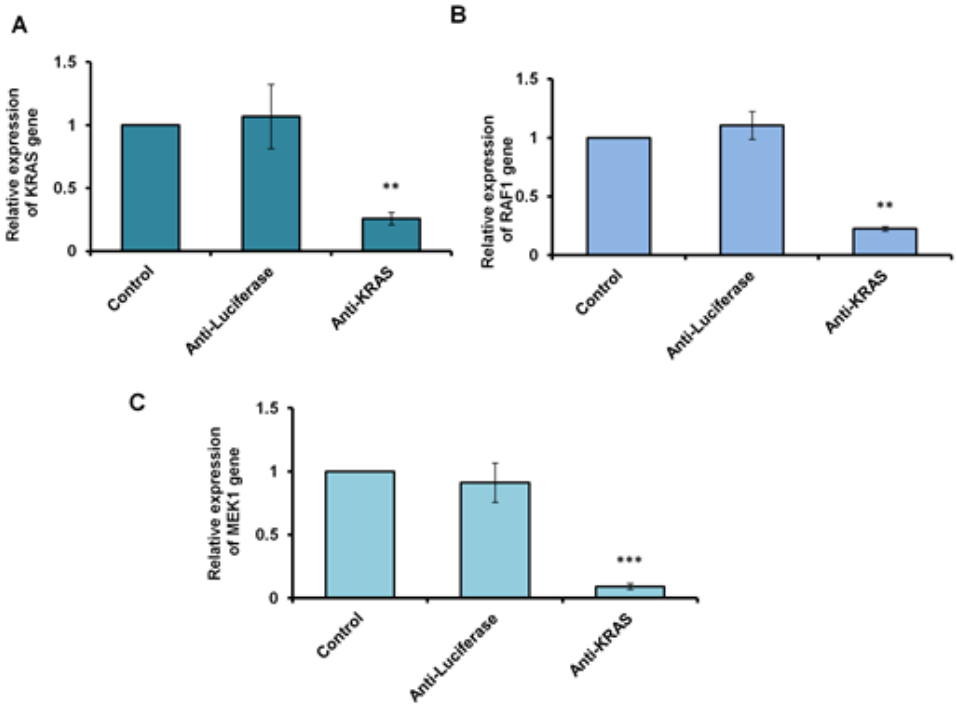

Figure 2. The knockdown efficiency of KRAS and relative genes expression. (A) Relative gene expression of KRAS in transfected HepG2 cells compared with control-transfected and no treated cells using qRT-PCR. (B and C) Relative gene expression of RAF1 and MEK1 in transfected HepG2 cells compared with control-transfected and nontreated cells using qRT-PCR. Error bars indicate the STD of three independent experiments. Student two-tailed t-test used for statistical analysis, (**) indicates *P* ≤0.01, and (***) indicates *P* ≤0.001.

Down regulation of KRAS increases the production of IL-6 and IL-8 in HepG2 cells.

ELISA assay was used to assess the produced levels of IL-6 and IL-8 from transfected cells to confirm the correlation of Ras gene expression in the secretion of inflammatory cytokines in cancer cells. Interestingly, the detected level of both IL-6 and IL-8 was increased by more than 250 pm/ml and 400 pm/ml at 48 hours post-transfected with siRNA antagonist *k-ras* compared with the control-transected cells. Notably, their levels were equal in control-transfected cells and cells transfected with siRNA antagonist luciferase (Figure 3A and B). This result indicates that transfection of HepG2 cells with siRNA antagonist *k-ras* resulted in the high production level of the inflammatory cytokine (IL-6) and IL-8, which incorporate with programmed cell death.

DISCSSION In normal cells, the RAS protein is involved in signaling pathways that facilitate gene transcription, which is required for cell growth and differentiation. Over-expression or mutation in the Ras gene can lead to uncontrolled cell growth and cancer development. Alteration of the Ras gene has been found in many types of cancer, including pancreatic, lung, thyroid, bladder, liver, and ovarian [12]. Notably, *k-ras* is a unique mutation of the RAS protein that frequently occurs in human cancer and is a primary downstream target of the epidermal growth factor (EGFR) pathway [24]. The activation of *k-ras* acts as sensors that initiate the activation of an array of signaling pathways, allowing the transmission of transduction signals from the cell surface to the nucleus, thus affecting cell differentiation, cell growth, and apoptosis [11]. In this way, we sought to target *k-ras* gene expression by using specific siRNA to confirm further the

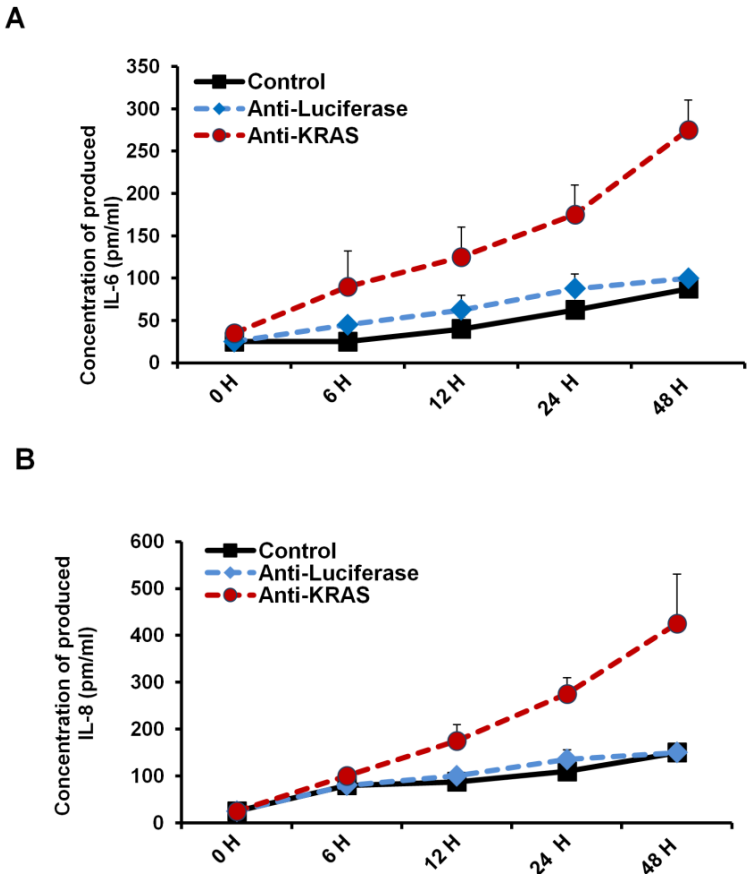

Figure 3. Levels of produced inflammatory cytokines in transfected HepG2 cells. (A) The concentration of IL-6 (pm/ml) produced in the fluid media of transfected HepG2 cells at the indicated time points. (B) The concentration of IL-8 in the culture media of transfected HepG2 cells simultaneously post-transfection. Error bars indicate the STD of four different replicates.

influential role of k-ras in HCC and the possible use of such siRNA in therapeutical strategy. The newly designed siRNA targets the mutated sequence within the coding sequences of the Ras gene. This strategy allows the specific regulation of cancer cells without affecting the expression of the wild-type Ras gene in normal cells, reducing the possible cytotoxicity of the exploiting siRNA. As expected, targeting the coding sequence of the Ras gene led to downregulating its downstream targets, including Raf-1 and Mek gene expression. This gene expression control of RAS/RAF/MEK signaling factors inhibits the essential pathway responsible for cancer cell proliferation.

A vital function of mutant RAS protein is the regulation of the MAPK pathway, which affects the proliferation of both normal and cancer cells [25]. Activation of *k-ras* stimulates *b-raf* and *Mek*, which activate genes involved in cell proliferation via the Erk transcription factor. The *k-ras* and b-raf mutations are crucial mediators in the EGRF signaling pathway and development in human liver cancer [14, 26]. Especially the RAS/RAF/MEK signaling pathways play a central role in hepatocarcinogenesis. Noteworthy, the k-ras gene is a necessary form of the Ras gene that acts as a critical primary related gene in the RAS/RAF/MEK signaling pathway. Activation k-ras gene point mutation mutations detected in many types of human cancers, such as pancreatic carcinoma (>80%), colon carcinoma (40-50%), and lung carcinoma (30-50%), and present at higher levels in the liver and breast [27]. Interestingly, we further confirmed the correlation between *k-ras* and RAF-MEK-ERK pathway connected with cell proliferation and interleukin secretion, including IL-6 and IL-8. These interleukins activate programmed cell death, resulting in cancer cell death [28]. Notably, IL-6 and IL8 are secreted by T cells and macrophages to stimulate the cellular immune response IL-6 and IL-8 act as regulatory factors of tumor-associated inflammation, and tumorigenesis has been well-identified in various cancer diseases, including HCC [29]. The cytokines, including IL-6 and IL-8, are multifunctional cytokine that plays a significant role in the response of hepatic epithelia to inflammation [30]. Our findings showed that IL-6 and IL8 significantly increased in HepG2 cells transfected with the siRNA antagonist k-ras mutant gene. These increasing levels of IL-6 and IL-8 can induce programmed cell death and control cancer cell proliferation.

Authors' contributions

Dina Ali performed the experiments. Adel A. Guirgis helped conceptualize experiments, interpret data. Hany Khalil designed the research plan, supervised overall research, provided, and interpreted data, organized, and wrote the manuscript.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of interest

All authors declare that there are no conflicts of interest.

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