

¹⁸⁸Rhenium Treatment Induces *DACT2* Expression in Hepatocellular Carcinoma Cells

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Received: 10/December/2020, Accepted: 19/April/2021

Abstract

Objectives: Epigenetic alterations, including any change in DNA methylation pattern, could be the missing link of understanding radiation-induced genomic instability. Dapper, Dishevelled-associated antagonist of β -catenin homolog 2 (*DACT2*) is a tumor suppressor gene regulating Wnt/ β -catenin. In hepatocellular carcinoma (HCC), *DACT2* is hypermethylated, while methylation status of its promoter regulates the corresponding expression. Radionuclides have been used to reduce proliferation and induce apoptosis in cancerous cells. Epigenetic impact of radionuclides as therapeutic agents for treatment of HCC is still unknown. The aim of this study was to evaluate epigenetic impact of ¹⁸⁸Rhenium perrhenate (¹⁸⁸ReO₄) on HCC cells.

Material and Methods: In this *in vitro* experimental study, HepG2 and Huh7 cells were treated with ¹⁸⁸ReO₄, receiving 55 and 73 Mega Becquerel (MBq) exposures, respectively. For cell viability measurement, live/dead staining was carried out 18, 24, and 48 hours post-exposure. mRNA expression level of β -Catenin, *Wnt1*, *DNMT1*, *DACT2* and *WIF-1* genes were quantified by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Then, possible regulatory impact of *DACT2* upregulation was investigated through evaluating methylation-specific PCR (MS-PCR).

Results: Results showed that viability of both cells was reduced after treatment with ¹⁸⁸ReO₄ at three time points post-exposure compared to the control groups. The qRT-PCR results showed that *DACT2* mRNA level was significantly increased at 24, and 48 hours post-exposure in HepG2 cells compared to the control group, while, no significant change was observed in Huh7 cells. Methylation pattern of *DACT2* promoter remained unchanged in HepG2 and Huh7 cells.

Conclusion: Treatment with ¹⁸⁸ReO₄ reduced viability of HepG2 and Huh7 cells. Although *DACT2* expression was increased after ¹⁸⁸ReO₄ exposure in HepG2 cells, methylation pattern of its promoter remained unchanged. This study assessed impacts of the ¹⁸⁸ReO₄ β -irradiation on expression and induction of *DACT2* epigenetic aberrations as well as the correlation of this agent with viability of cells.

Keywords: DNA Methylation, Epigenetics, Hepatocellular Carcinoma, Radionuclide

Cell Journal (Yakhteh), Vol 24, No 5, May 2022, Pages: 215-221

Citation: Asadian S, Piryaeei A, Farzaneh Z, Aziz Kalantari B, Azad M, Moghbeli Nejad S, Davarpanah MR, Mohamadi M, Shpichka A, Gheibi N, Timashev P, Vosough M. ¹⁸⁸Rhenium treatment induces *DACT2* expression in hepatocellular carcinoma cells. Cell J. 2022; 24(5): 215-221. doi: 10.22074/cellj.2022.7894. This open-access article has been published under the terms of the Creative Commons Attribution Non-Commercial 3.0 (CC BY-NC 3.0).

Introduction

Hepatocellular carcinoma (HCC) is a common malignancy, globally (1). Tumorigenesis is followed by deviations of gene expression and protein function initiated by genetic and epigenetic modifications. The molecular pathways contributing to hepatocarcinogenesis are a multi-stage process involved in progressive accumulation

of molecular aberrations determining different molecular, cellular and histopathological events (2).

Activation of the Wnt/ β -catenin signaling pathway is commonly associated with initiation and progression of HCC, colorectal cancer and other different types of cancer (3). The association of alterations in Wnt signaling

pathway with cancer development was signified after reporting that highlighted the activation of *int1* (*Wnt1*), either by pro-viral insertion into the *Wnt1* locus or transgenic overexpression in mice model, resulted in mammary malignancies (4).

The Wnt/ β -catenin pathway regulates cell proliferation and plays a crucial role in the carcinogenesis of HCC. Genetic mutations or abnormal activation of the Wnt/ β -catenin pathway are key players in the tumor development within up to 50% of HCC cases. Mutations of the *Catenin* gene are thought to be the prominent genetic aberration initiating development of HCC (5).

The first proof of crucial impact of epigenetic changes in cancer development and progression was reported in 1983, by Feinberg and Vogelstein. They showed shifted methylation pattern of genes in colorectal tumors (6). Epigenetic alterations play crucial roles in the pathogenesis of many human diseases, including cancer (7), particularly HCC (8). In this regard, recent studies showed that methylation in the promoter region of *dapper*, dishevelled-associated antagonist of β -catenin homolog 2 (*DACT2*) gene, as an antagonist of β -catenin, was correlated with loss or reduction of *dapper*, while Wnt inhibitory factor 1 (*WIF-1*) promoter hypermethylation activates Wnt/ β -catenin pathway (9). *Dapper* was identified by screening proteins that interact with dishevelled, a critical factor in the Wnt signaling. *Dapper* and dishevelled were co-localized intracellularly and formed a complex with Axin, GSK3 and β -catenin to continue the pathway (10). Researchers found that *DACT2* expression was downregulated in certain colorectal cancers (11). Similarly, it was reported that mRNA expression of *DACT2* was downregulated in human HCC (12). Therefore, *DACT2* was considered as a tumor suppressor gene in many types of tumors. Researchers demonstrated that tumor size was larger (>5 cm) in HCC patients with downregulated *DACT2*, compared to those with high *DACT2* expression. Thus, this gene may play a substantial role in the growth and development of HCC cells (13). Several studies evaluated potential correlation of mRNA expression level with promoter methylation of *DACT2* in different tumors (14). Likewise, to the previous reports in different types of cancers, *DACT2* expression was regulated by hypermethylation of the corresponding gene promoter. The promoter hypermethylation might be a crucial mechanism of *DACT2* gene silencing transcriptional level in HCC cells (13).

WIF-1 is an endogenous antagonist for Wnt. It inhibits Wnt pathway through binding to Wnt proteins in the extracellular space (15). Recent studies showed association of *WIF-1* promoter hypermethylation with the corresponding gene silencing in HCC (16). This loss of gene expression could be restored after treatment with epigenetic modification drugs (17).

Among the targeted therapies, using radionuclides introduced as a potential intra-tumoral radiation-based treatment approach for HCC. In this approach,

administration of radionuclides into the hepatic artery targets cancer cells within tumor mass, whereas the non-cancerous surrounding tissue remains unaffected (18, 19). In this regard, various radionuclides have been reported including Yttrium-90 microspheres, Rhenium-188 lipiodol, Iodine-131 lipiodol, Rhenium-188 microspheres, Holmium-166 chitosan and Holmium-166 microspheres for intra-arterial therapy of liver carcinoma (20).

Recent *in vivo* studies demonstrated that radionuclides could have a remarkable impact on the epigenetic status, particularly in DNA methylation pattern (21). Notably, continuous and chronic exposure induced epigenetic changes such as non-coding area hypermethylation associated with genomic instability up to 20 consecutive passages post-irradiation (22). It has been suggested that an external epigenetic driver could be involved in this phenomenon, such as ROS-radicals, methylation changes or microRNA mediated signaling (23). ROS production due to ionizing radiation is linked with alterations in DNA methylation pattern (24). Hydroxyl radical-induced DNA damage (25) have been shown to induce DNA hypomethylation by interfering with DNA methyltransferases (DNMTs) and therefore resulting in whole genomic hypomethylation (26). In addition, oxidative stress induced by ROS can induce gene silencing by abnormal hypermethylation of promoter regions in tumor suppressor genes. Thus, it might lead to cancer progression.

Epigenetic alterations are dynamic and usually work as an adaptation mechanism to different changes of environmental factors. Even though there is growing evidences on the importance of epigenetics and biological processes induced by radiotherapy in various cancer types including HCC, specific epigenetic effects of radionuclides on *DACT2*, as an important inhibitor of Wnt/ β -catenin signaling, are not revealed completely at the molecular level.

In this study, we investigated apoptosis induction capacity of ¹⁸⁸Rhenium perrhenate (¹⁸⁸ReO₄) on HepG2 and Huh7 cells, as well as normal fibroblasts. After 18, 24 and 48 hours post-irradiation by ¹⁸⁸ReO₄, cell viability was measured through live/dead assay. Gene expression of particular genes and promoter methylation pattern were evaluated to investigate possible epigenetic changes.

Material and Methods

Ethical approval

The Ethical Committee of Royan institute (IR.ACECR.ROYAN.REC.1397.052) approved this study.

Cell culture and treatment

In this *in vitro* experimental study, HepG2 and Huh7 cells were obtained from Royan Cell Bank (Royan Institute, Iran). The cells were cultured in high-glucose Dulbecco's modified Eagle's medium (HGDMEM, Gibco, USA) at

37°C in a humidified cell culture incubator with 5% CO₂. The culture medium was enriched with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM non-essential amino acids and 1% penicillin/streptomycin (Pen/Strep, all from Gibco, USA). Cells were sub-cultured by trypsin/EDTA (0.25%, Gibco, USA). The culture media were refreshed every day. Expression level of all genes was normalized to the expression levels at time zero. All viability values were presented in percentage and normalized to the viability values at the time zero.

Cell viability assay

After 18, 24 and 48 hours post-treatment with 55 and 73 MBq of ¹⁸⁸ReO₄, HepG2 and Huh7 cells (initial cell seeding number: 2500 cells) were suspended in PBS and mixed with LIVE/DEAD[®] viability/cytotoxicity kit (Invitrogen, USA), consisting of live/dead staining solution (0.2 μM calcein-AM and 0.1 μM ethidium homodimer-1). The cancer cells were incubated for 20 minutes at room temperature with the reagent. Cell viability was shown by green or red fluorescence labelled cells representative for live and dead cells, respectively. Live and dead cells were observed by counting green and red fluorescent signals using fluorescence microscope (Olympus, Japan).

Image analysis was conducted to preliminary recognition of pixels with the given color channel intensity (red or green), and then counting live and dead cells using ImageJ (Imagej.nih.gov). To validate cell viability measurements, non-overlapping images (three pictures) from a coverslip were used to analyze mean value for each single coverslip, which represented one experimental measurement. Number of experiment repeats is indicated as "n". The student's two-tailed t test, ANOVA and post hoc Bonferroni test were used in terms of statistical analysis.

Quantitative reverse transcription–polymerase chain reaction

In order to study expression levels of *DNMT1*, *Wnt1*, *β-Catenin*, *WIF-1* and *DACT2* using quantitative reverse transcription-polymerase chain reaction (qRT-PCR), we designed primers. mRNA expression level of the genes were quantified in HepG2 and Huh7 cells that treated with 55 and 73 MBq of ¹⁸⁸ReO₄ and the control groups. To evaluate Wnt/β-catenin signaling pathway in HepG2 cells, qRT-PCR was performed for *β-Catenin*, *Wnt1*, *DNMT1*, *DACT2* and *WIF-1* genes. RNA extraction was performed using TRIzol (Invitrogen[®], USA), and cDNA was synthesized using PrimeScript[™] Reverse Transcriptase Kit (Takara Bio, Japan) according to the manufacturer's instructions. qRT-PCR reactions were performed using a real-time PCR system (Applied Biosystems StepOne instrument, USA) using SYBR Green Master Mix (Takara Bio) and the results were analyzed by StepOne software (Applied Biosystems). The samples were collected from three independent biological replicates for each group. Finally, mRNA expression level of each gene was

normalized to *GAPDH* and calculated relative to HepG2 and Huh7 in the adherent culture. Analysis was performed by the comparative CT Method, 2^{-ΔΔCt}. The primers are listed in the Table 1.

Table 1: List of the primers used for qRT-PCR and MS-PCR in this study

Gene	Primer sequence (5'-3')
<i>GAPDH</i>	F: CAATGACCCCTTCATTGACC R: TGGAAGATGGTGATGGGATT
<i>β-Catenin</i>	F: CATCTACACAGTTTGTGCTGCT R: GCAGTTTTGTGAGTTCAGGGA
<i>Wnt1</i>	F: GGGCATCGTGAACATAGCCTCCTCC R: CGGCGGAGGTGATTGCGAAGATAAA
<i>DNMT1</i>	F: CCGACTACATCAAAGGCAGC R: AGGTTGATGTCTGCGTGGA
<i>DACT2</i>	F: GGCTGAGACAACAGGACATCG R: GACCGTCGCTCATCTCGTAAAA
<i>WIF-1</i>	F: TATGGATCGATGCTCACCAG R: CAGAGGGACATTGACGGTTG
<i>DACT2</i>	F: GATTTTAGTTTATTTTGGCGATTGTC R: CACATCTCCCGAACAAAATCCCG
<i>DACT2</i>	F: TAGATTTTAGTTTATTTTGGTGATTGTT R: TCCACATCTCCCAAACAAAATCCCA

qRT-PCR; Quantitative reverse transcription-polymerase chain reaction and MS-PCR; Methylation-specific PCR.

Methylation-specific PCR

Genomic DNA from test and control group of HepG2 and Huh7 cells were extracted using the Proteinase-K method. After chloroform/phenol extraction, DNA precipitation was performed in ethanol. DNA was dissolved in low Tris/EDTA (TE) buffer and stored at -20°C (27). Genomic DNA was extracted from treated and untreated cells, followed by reacting with bisulfite to perform MS-PCR. The MS-PCR primers were designed considering the genomic sequences, flanking the presumed transcription start site (TSS). The primer sequences were oligo-synthesized (Invitrogen, USA) in order to perform MS-PCR for *DACT2* and detect bisulfite-induced changes affecting unmethylated and methylated alleles. The *DACT2* primers were used in MS-PCR listed in the Table 1.

Statistical analyses

Statistical analysis was performed using SPSS version 20 (IBM Co., USA) and PRISM 6.0 software package (GraphPad Software

Co., USA). Statistical analysis was performed using one-way ANOVA and independent-sample t test. The $P < 0.05$ was considered statistically significant. Data was presented as mean \pm standard deviation (mean \pm SD). Samples were collected from three independent biological replicates.

Results

Cell viability after treatment of HepG2 and Huh7 cells with $^{188}\text{ReO}_4$

To quantify cell viability, HepG2 and Huh7 cells were treated with 55 and 73 MBq of $^{188}\text{ReO}_4$. They were evaluated at three time points, including 18, 24 and 48 hours post-exposure. Then, live/dead assay was performed as mentioned in material and methods. As shown in Figure 1A, 55 MBq of $^{188}\text{ReO}_4$, produced a reasonable impairment of cell viability, and viability at the three time points were 66.45%, 62.73% and 49.92%, respectively, as shown in Figure 1B. For Huh7 cells, treatment with 73 MBq of $^{188}\text{ReO}_4$ resulted in reduced viability up to 42%, 50%, and 54% at the three time points. After 48 hours exposure with 55 MBq of $^{188}\text{ReO}_4$, statistically significant cell death occurred in comparison with the control group ($P < 0.05$). This data suggested that exposure to $^{188}\text{ReO}_4$ made a significant impact on the cell viability in the both cell lines.

Quantitative reverse transcription-polymerase chain reaction

Downregulation of *DNMT1* was observed at all of the three time points; however it was significant only at the 18 hours post-exposure compared to the control group (Fig.1C). mRNA expression of β -catenin was upregulated after treatment with $^{188}\text{ReO}_4$, compared to the control group. The mentioned upregulations were significant at the 18 and 24 hours post-exposure. There was not significant difference in the expression of β -catenin between the treated HepG2 cells and control group 48 hours after treatment (Fig.1D). To further analyze impact of $^{188}\text{ReO}_4$ on Wnt/ β -catenin signaling pathway, relative mRNA expression of *Wnt1* was measured by qRT-PCR. Data showed no significant difference between the treated cells and control HepG2 cells at all of the time points (Fig.1E). qRT-PCR data showed a significant upregulation in *WIF1* level, 48 hours post-treatment compared to the control cells (Fig.1F), while there was not any significant change in the other time points. Expression of *DACT2* was upregulated at all of the time points. However, in HepG2 cells the mRNA expression was significantly higher than control group, 24 and 48 hours after exposure (Fig.1G).

Methylation status of *DACT2* promoter in HCC cell lines didn't change after exposure

The results showed that *DACT2* transcript was upregulated in HepG2 cells, 24 and 48 hours after treatment with 55 MBq of $^{188}\text{ReO}_4$ (Fig.1G). To evaluate whether upregulation of *DACT2* is correlated with epigenetic alteration of the promoter methylation status

after treatment with $^{188}\text{ReO}_4$, we carried out MS-PCR. The results indicated that *DACT2* promoter in the HepG2 cells remained methylated at the 18, 24, and 48 hours post-treatment with $^{188}\text{ReO}_4$ (Fig.1H, I). The same experiment was performed for Huh7 cells treated with $^{188}\text{ReO}_4$ at the three time points, resulted in the same findings. These data proposed that mRNA expression level changes of *DACT2* are not associated with epigenetic changes in methylation status of its promoter.

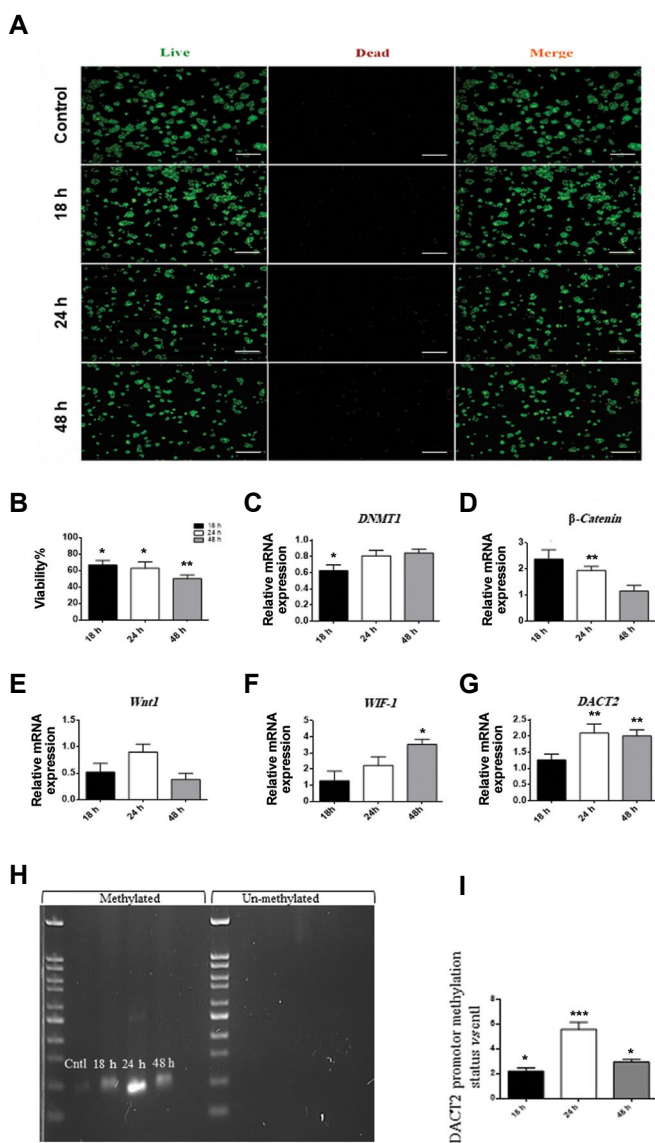


Fig.1: Molecular modifications after treatment of cells with $^{188}\text{ReO}_4$. **A.** Live/dead cell viability assay of HepG2 cells treated with 55 MBq $^{188}\text{ReO}_4$, and untreated HepG2 cells (control group). The cells were evaluated at 18, 24 and 48 hours after treatment. The live and dead cells were visualized in green and red fluorescence, respectively (scale bar: 500 μm). **B.** Viability percent vs. control after treatment with 55 MBq at three time points 18, 24 and 48 hours. **C-G.** qRT-PCR analysis for *DNMT1*, β -catenin, *WNT1*, *WIF-1* and *DACT2*, to evaluate relative mRNA expression in HepG2 cells treated with 55 MBq of $^{188}\text{ReO}_4$ vs. control after 18, 24 and 48 hours post-exposure. **H.** MS-PCR test results. Methylation status of the *DACT2* promoter was evaluated by MS-PCR in HepG2 cells after 18, 24, and 48 hours post-exposure. **I.** Promoter methylation status of *DACT2* was quantified by ImageJ in HepG2 cell line at three time points after treatment. Data are presented as the mean \pm SD, $n=3$ (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$). h; Hour.

Exposure impact on normal cells and Huh7 cells

To evaluate the impact of treatment with $^{188}\text{ReO}_4$ on normal cells, the same experiment was performed on human dermal fibroblasts (HDF). Viability of HDF cells did not show any significant difference after treatment with various exposures at the three time points. Percentage of dead cells are also illustrated at different time points (Fig.2A).

DACT2 mRNA expression did not show any significant change in the three time points compared to the control group of Huh7 cells. β -catenin expression in Huh7 cells showed a similar trend to HepG2 cells, however, 48 hours post treatment, downregulation was significant compared to the control group (Fig.2B).

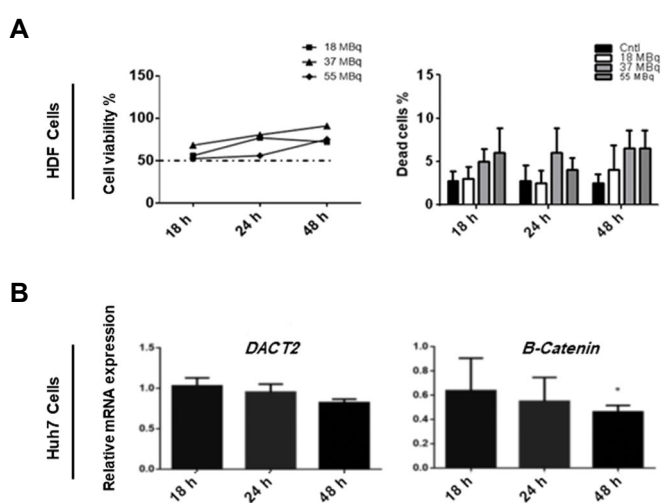


Fig.2: The exposure impact on normal cells and Huh7 cells. **A.** Evaluation of viability after treatment with $^{188}\text{ReO}_4$ in HDF cells. Viability of the HDF cells were visualized and compared to the control (untreated) cells after treatment with 18, 37 and 55 MBq $^{188}\text{ReO}_4$ at 18, 24 and 48 hours. Mean viability percent vs. control after treatment with 18, 37 and 55 MBq in HDF cells after 18, 24 and 48 hours exposure. Percentage of the dead cells treated with the same condition were presented here. Data are expressed as the mean \pm SD, n=3 (*; $P < 0.05$) vs. control group. **B.** The bar graphs show quantification of the qRT-PCR analysis for *DACT2* and β -catenin in Huh7 cells treated with $^{188}\text{ReO}_4$ and received 73 MBq vs. control after 18, 24 and 48 hours post-exposure. Data are presented as the mean \pm SD, n=3, (*; $P < 0.05$)

Discussion

DNA methylation status change is a crucial feature of epigenetic modification, which initially occurs in the CpG islands of gene promoter regions. Activation of multiple DNMTs are required to establish and maintain DNA methylation patterns (28). Some studies showed that DNA methylation response secondary to irradiation is the same to the common biological stimulations (29). In the current study, we showed that mRNA expression level of *DACT2* was upregulated in HepG2 cells, at the 24 and 48 hours post-exposure to $^{188}\text{ReO}_4$. These results were in correlation with decreased cell viability at the 18, 24, and 48 hours post-exposure. Therefore, *DACT2* may play critical role in the viability of cancer cells and progression

of HCC (13). Aberrant activation of Wnt signaling is a significant cause for initiation and progression of cancer, which could be originated from genetic or epigenetic changes (30). Frequency in methylation status of Wnt signaling antagonists proposes a vital role in the activation of this pathway during carcinogenesis (31). *DACT2* accelerates dishevelled (Dvl/Dsh) degradation in the lysosome-dependent pathway. This inhibits LEF1 binding to β -catenin.

Restoring expression of *DACT2* induces transcriptional activation of T cell factor-4 and the downstream Wnt signaling, which plays a suppressive role for them (32). *DACT2* inhibits cell proliferation, induces breakage in G2-M phase in cell lines and inhibits tumor growth in the xenograft nude mice (11). *DACT2* expression was silenced by hypermethylation of its promoter in HCC, suggesting that the transcriptional silencing of *DACT2* may be one of the main factors in the progression of HCC (15). Moreover, previous studies showed another antagonist of Wnt signaling, *WIF-1*, which is downregulated and hypermethylated in the HCCs compared to the normal liver tissue (33).

Possible association of *DACT2* expression with radionuclide therapy has not yet been studied in human cancer. Studies highlighted implication of such therapies that restore tumor suppressive function of *DACT2* and *WIF1* in HCC patient; however, therapeutic impact of the radionuclides on HCC cells as well as the increased expression of *DACT2* and *WIF-1* have been remained to be explored. The role of *DACT2* as a tumor suppressor, its downregulation in cancer and correlation of *DACT2* expression with the methylation status of its promoter have been studied in colorectal cancer. In colon cancer, restoring *DACT2* expression repressed malignant cell growth by inducing apoptosis and proliferation inhibition both *in vitro* and *in vivo* models (11). Additionally, aberrant promoter hypermethylation of *DACT2* was reported in the other types of cancer, indicating significant reduction of *DACT2* expression (34). These findings led us to study potential correlation of expression level and promoter methylation status of *DACT2* gene in HCC cells after treatment with radionuclides.

It has been reported that radiation could induce epigenetic alterations post-irradiation (35, 36). Particularly, reactive oxygen species (ROS) production is associated with alterations in DNA methylation patterns. Moreover, ROS derivatives contribute to DNA hypomethylation by interfering with the DNMTs and therefore resulting in decreased methylation (26).

The present study was conducted to investigate possible correlation of β -irradiation with $^{188}\text{ReO}_4$, as a novel therapeutic agent, on HCC cells and remodeling of *DACT2* promoter methylation status. This study showed that viability of HepG2 and Huh7 cells was declined noticeably after exposure to β -irradiation. We showed for the first time that *DACT2* and *WIF-1* were upregulated in HepG2 cells, 24 and 48 hours after treatment. This

data suggested that viability changes might be due to the impaired Wnt/ β -catenin pathway. We carried out qRT-PCR to determine whether $^{188}\text{ReO}_4$ induced upregulation of *Wnt1* and β -catenin expression levels. Expression of *Wnt1* was significantly upregulated in HCC cells and it had a key role in the survival of the HCC cells (37). Our data showed no significant reduction of *Wnt1* and β -catenin expression in mRNA level. Further investigations showed that expression level of *DNMT1* was not changed significantly in HepG2 cells after treatment with $^{188}\text{ReO}_4$, compared to the control group. Additionally, to find out possible correlation of *DACT2* upregulation and epigenetic alterations on its promoter after treatment with $^{188}\text{ReO}_4$, promoter methylation status of the *DACT2* was evaluated by MS-PCR in the both cell lines. Our data suggested that *DACT2* upregulation is not associated with promoter hypomethylation after exposure to $^{188}\text{ReO}_4$ in HepG2 and Huh7 cells.

Comparison of our results with previous studies showed that radiation did not significantly change the activity of Wnt/ β -catenin pathway (38). However, previous studies showed that low-dose radiation induced upregulation of *Wnt1*, *Wnt3a*, *Wnt5a* and β -catenin (39). In our study, there were significant β -catenin upregulations at 18 and 24 hours post-exposure of HepG2 cells while there was no change in *Wnt1* expression. Few studies showed that sirtuin 2 (SIRT2) in response to radiation-induced stress directly interacted with β -catenin and inhibited Wnt signaling (40). This study did not show any correlation between epigenetic status of *DACT2* promoter after treatment with $^{188}\text{ReO}_4$ and alteration in mRNA expression level in the both lines.

Conclusion

$^{188}\text{ReO}_4$ treatment reduced viability of HepG2 and Huh7 cells. This exposure increased expression of *DACT2* in HepG2, but it did not affect epigenetic status of its promoter. However, Huh7 cells did not show any change in the *DACT2* expression level and epigenetic status of the corresponding gene promoter. Further work is needed to find the exact impact of β -irradiation on epigenetic modifications of the Wnt/ β -catenin pathway.

Acknowledgments

The authors would like to express their gratitude to their colleagues at Regenerative Medicine Department of Royan Institute (Tehran, Iran). "The study was partly supported by i. National Cancer Control Charity Foundation, registration number 41476, Tehran, IRAN, ii. Ghazvin University of Medical Science, and iii. The Ministry of Science and Higher Education of the Russian Federation within the framework of state support for the creation and development of World-Class Research Centers "Digital biodesign and personalized healthcare" (N. 075-15-2020-926). The authors declare no conflict of interest.

Authors' Contributions

S.A.; Performed the experiments, as part of her thesis,

drafting the manuscript and drawing the figures. A.P., Z.F., M.A., A.S., P.T., S.M.N.; Helped and involved in study design, analyses and drafting the manuscript. B.A.K., M.R.D., M.M.; Prepared the radionuclide and involved in study design and revising the manuscript. P.T., N.G., M.V.; Designed the study, performed the analysis, writing and revising the manuscript as well as proofreading. All authors read and approved the final manuscript.

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