Malaysian Journal of Medicine and Health Sciences (eISSN 2636-9346)

ORIGINAL ARTICLE

Analyses of *Rac1* siRNA knockdown for Nasopharyngeal Carcinoma/HK1 Cell Line

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ABSTRACT

Introduction: Patients with Nasopharyngeal carcinoma (NPC) usually diagnosed at advanced cancer stage and recurrent case. *Rac1* have become an emerging therapeutic target for metastasis cancer. This gene is critically involved in cell polarization and reactive oxygen species-mediated cell killing. This study aims to investigate the *Rac1* activities in NPC/HK1 cell line using siRNA approach and evaluate the calcium deposition profile. **Methods:** The NPC/HK1cells were transfected with *Rac1*-siRNA (si*Rac1*) at concentrations of 50nM, 100nM and 200nM for 24 hours and stained with alizarin red s for calcium mineralization profile. Levels of *Rac1* gene expression were measured via qRT-PCR followed by the time dependent assessment for 24, 48 and 72 hours. **Results:** Findings revealed that si*Rac1* concentrations of 200nM (p-value <0.02) and 100nM (p-value <0.016) had significant *Rac1* suppression while 50nM (p-value <0.076) had the least suppression. On the other hand, from alizarin red S staining showed no significant changes for calcium mineralization activity on treated and control cells. However, si*Rac1* treated cells at 200nM showed presence of intracellular organelle swelling and loss of membrane integrity in 70% of the cells. This observation could possibly be linked to early sign of necrosis activity, hypoxia and disruption in intracellular calcium influx. **Conclusion:** This study suggest that *Rac1* gene suppression might be involved in disruption of calcium deposition and reactive oxygen species-mediated NPC/HK1 cell killing. Further insight on the *Rac1* molecular mechanism are needed to understand its potential role as therapeutic target for NPC.

Keywords: Nasopharyngeal carcinoma, *Rac1*, Calcium mineralization in cancer, siRNA therapeutics, Reactive oxygen species-mediated cell killing

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INTRODUCTION

Nasopharyngeal carcinoma (NPC) is one of the leading head and neck cancer which are predominantly in Asian countries (1,2). Presently, nasopharyngeal malignancies are usually diagnosed at late stage. NPC stage IV patients have a poor prognosis, even after receiving an effective chemotherapy and associated with recurrence cases (3, 4). Therefore, the discovery on potential molecular biomarkers are useful for effective diagnostic, prognostic tools and even in therapeutic strategies (5). A number of critical regulatory pathways in NPC that contribute to the malignancy phenotype, resistance behavior and recurrence cases were still not clear. Many studies have reported that upregulated level of *Rac1* expression has been associated with the development of numerous human cancers (6) and associated with tumors malignancy phenotype (7, 8).

Rac1 is a known member of the family of the Rho GTPase which are involved in the malignant transformation events such as invasion, metastasis and angiogenesis (7). These cellular pathways are regulated via reactive oxygen species (ROS) and calcium (Ca^{2+}) influx activities (8, 9). This interaction of ROS and Ca^{2+} stimulates signaling cascades in the cells promoting proliferation and subsequent metastasis (10). Cancer cell metastasis relies on the actin cytoskeleton activities via the filopodia and lamellipodia (11). The actin dynamics that necessitate the contractility and directional movement stabilization of cells is regulated via Ca^{2+} signaling and kinases (12, 13). Furthermore, this phenomenon also link to the ROS activities via ROS-dependent HIF-1α and Rho GTPases in the mitochondrial cristae and intermembrane spaces (14) which drives the migration activities (15-18).

Therefore, *Rac1* might present as potential therapeutic target in cell polarization and reactive oxygen species-mediated cell killing mechanisms for NPC (19).

This study investigated the suppression of *Rac1* on NPC by using small interfering RNA (siRNA) approach. The optimization work on concentration and time dependent suppression expressions of *Rac1* were studied along with calcium deposition profile. Understanding of *Rac1* activities on NPC cell line are crucial in anticipation of developing an efficiency therapeutic target against NPC.

MATERIALS AND METHODS

Cell culture

NPC/HK1cell line was kindheartedly given by Professor, George Tsao of University of Hong Kong (20) and 105 cells was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco, Life Technologies, Carlsbad, CA, USA).

si*Rac1* concentration and time dependent assessment

The si*Rac1* obtained from ThermoFisher Stealth RNAi was transfected into 105 NPC/HK1 cells in 12 well plates using SureFect transfection reagent (SABioscience) according to manufacturer's instruction. The concentration dependent *Rac1* suppression were assessed at 50, 100 and 200nM for 24 hours (21-23). For time dependent suppression si*Rac1* with 100nM were further studied in 105 NPC/HK1 using 24 well culture plates at 24, 48 and 72 hours (22). The untreated cells with si*Rac1* were used as *Rac1* expression control. All the experiments were performed in triplicates. The RNeasy extraction kit (Qiagen), QuantiNova cDNA synthesis kit (Qiagen) and QuantiNova SYBR Green PCR kit (Qiagen) were used to prepared the samples following the manufacturer's instruction. The gene expression was assessed using StepOnePlus real time PCR system (Applied Biosystems) with a set of *Rac1* primers: 5'-GCCAATGTTATGGTAGAT-3' and 5'-GACTCACAAGGGAAAAGC-3'. A set of GAPDH primer: 5'-AACGGATTTGGTCGTATTG-3' and 5'-GCTCCTGGAAGATGGTGAT-3' used as an endogenous control (23). GAPDH was used for normalization of the data of the expressed gene, because of its constant expression and has previously been used as endogenous control in many NPC cell lines including NPC/HK1 cells (24). VanInsberghe et al., (2018) has also used GAPDH as endogenous control in a related recent experiment (25).

Alizarin Red S Staining

The protocol was carried out according to Eapen A., et al. (26). Briefly, the cells were washed and fixed with 4% formaldehyde at room temperature for 1hr and stained with 2% solution of Alizarin Red S. The images were taken at x 20 objective under phase contrast microscope (Olympus CKS40).

Statistical Analysis

Statistical analyses were performed with SPSS v16.0 (SPSS, Chicago, IL, USA). The average of the experimental groups were compared with that of control group using paired Student's t-test to compare the relative expression of *Rac1*. Any value less than $p < 0.05$ was regarded as being statistically significant.

RESULTS

si*Rac1* Transfection Profiles

After 24 hours of si*Rac1* transfection into HK1/NPC cells at varying si*Rac1* concentrations, we observed differential suppression of *Rac1* mRNA expressions from control, 50, 100 to 200nM (Fig. 1). Significant suppression amongst the three treated replicates were achieved at si*Rac1* 200nM with 80% followed by 100nM with 65% and 50nM with 50% mRNA suppression compared to control. Both si*Rac1* 200nM and 100nM have p value < 0.05 (p value < 0.02 and p value <0.016 respectively) while 50nM has p-value < 0.076 (Fig.1). A consistent *Rac1* suppression in NPC/HK1 cells was observed at the three time points of 24, 48 until 72 hours as represented in the histogram with all having p value < 0.05 (Fig. 2).

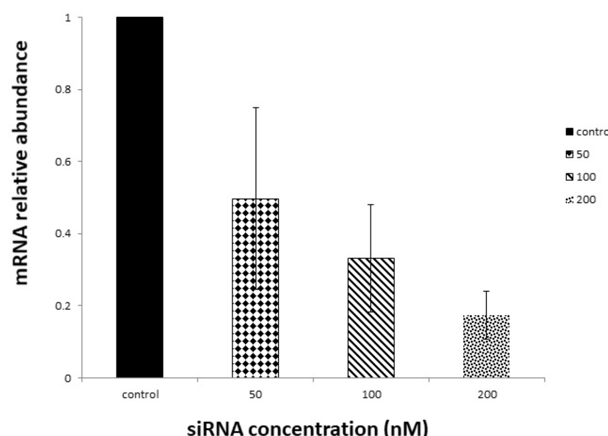

Figure 1: si*Rac1* Concentration Dependent Suppression Profile. Histogram from triplicate data showed 80% suppression of *Rac1* was achieved at si*Rac1* concentration 200nM (p <0.02), 65% for 100nM si*Rac1* (p <0.016) and 50% for 50nM si*Rac1* (p <0.076). Experiments were carried out in triplicates.

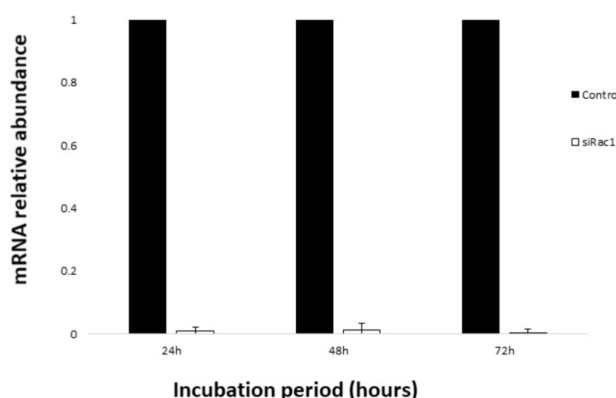

Figure 2: si*Rac1* Time-Dependent Suppression Profile. *Rac1* suppression on NPC/HK1 cells at 100nM were consistent at the three time points starting 24, 48 until 72 hours. The histogram represents triplicate experiments with p value < 0.05.

Alizarin Red S Staining

The evaluation of calcium mineralization profile of NPC/HK1cells using alizarin red s, revealed no significant changes in the staining intensity of si*Rac1* 50, 100 and 200nM transfected cells when compared to control cells (Fig. 3). si*Rac1* treated cells at 200nM showed presence of intracellular organelle swelling and loss of membrane integrity associated early sign of necrosis activity (Fig.3d) compared to control NPC/HK1 cells (Fig. 3a). Each image represents quadruples the experiments with magnification at 20 x objective and tool bar 100µm.

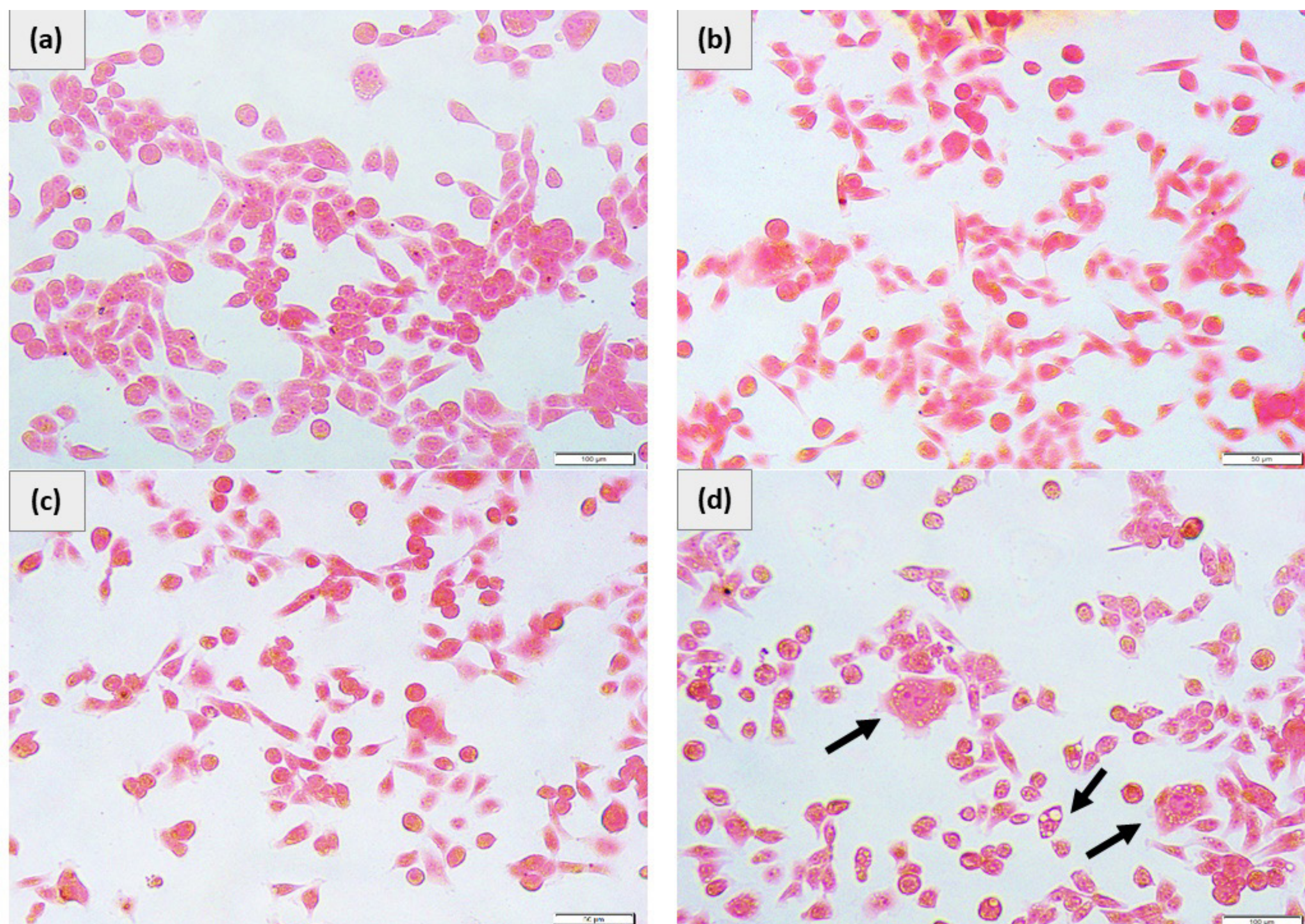

Figure 3: Alizarin Red S Staining of si*Rac1* treated NPC/HK1cells. There is no clear difference in calcium mineralization activities between (a) control and si*Rac1* transfected cells at concentrations of (b) 50nM, (c) 100nM and (d) 200nM. However, si*Rac1* treated cells at 200nM showed intracellular organelle swelling and loss of membrane integrity sign (Fig. 3c and 3d) that could be associated with the necrotic-like phenotype (black arrow). Each images represent quadruples the experiments with magnification at 20 x objective and tool bar 100µm.

DISCUSSION

Our findings revealed that *Rac1* suppression in NPC/HK1cells was achieved between 50% - 80% at 100 and 200nM siRNA (Fig. 1). siRNA concentration plays are crucial in determination of targeting specificity and extortionate siRNA concentration analogous the off target effects (27, 28). Further investigation on siRNA concentration at 100nM showed consistent suppressions between the time periods from 24-72 hours (Fig. 2). Note that, the suppression of *Rac1* was achieved near 100% when using smaller culture systems in the 24 well plate (same transfection concentration and cell number) (Fig. 2) compared to 12 well plate (Fig. 1). The efficiency of cellular uptakes of si*Rac1* could also be influenced by available space and seems more effective with smaller spaced culture systems. The si*Rac1* crest effects on transfected cells have been reported to be between 36–48 hours and starts to lower at 96 hours depending on the cell line model (29, 30).

From the alizarin red s staining profiles revealed that si*Rac1* concentrations treatment showed no clear sign on changes of calcium mineralization (Fig. 3) compared to control NPC/HK1 cells (Fig. 3a), notwithstanding distinctly the si*Rac1* treated cells showed intracellular organelle swelling and loss of membrane integrity sign (Fig. 3c and 3d). Generally, this observation is an early sign of necrosis that could be linked to prolonged periods of severe hypoxia, an aggravated cellular re-oxygenation injury that associated with increases cellular ROS formation and Ca2+ influx/deposition activities (31-34). By contrast, an emerging evidence suggests that the coordination between Ca2+ and ROS is disrupted in cancerous cells causing resistance to apoptosis (35-38). This study suggests that the mechanistic activities

of the necrosis could be triggers by interchange signals from cellular Ca2+ and ROS activities. However, further study on the necrosis marker are needed to validate these findings.

Rac1 gene plays crucial role in controlling the cellular redox state (39) thus suppression of this gene could be linked with reactive oxygen species-cell killing mechanisms. This phenomenon could be explained by the activities of ROS and Rho GTPase which are also known as the switches for killing (40-43). Thus, *Rac1* suppression could involve positive cellular regulation events of Rho proteins in NPC/HK1 cells, thus present as potential therapeutic target.

CONCLUSION

In conclusion, this study suggests that *Rac1* showed a possible molecular switch for necrosis in targeting the reactive oxygen species-mediated NPC/HK1 cell killing. Further studies are needed to understand the necrosis molecular mechanism of *Rac1* functioning as a therapeutic target for NPC.

ACKNOWLEDGEMENTS

The authors would like to thank Universiti Sains Malaysia (Short Term Research Grant: 304/CIPPT/6315073) for sponsoring this work.

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