Transcript-level regulation of MALAT1-mediated cell cycle and apoptosis genes using dual MEK/Aurora kinase inhibitor “BI-847325” on anaplastic thyroid carcinoma

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Abstract

Background Anaplastic thyroid carcinoma (ATC) is the most lethal malignancy in thyroid carcinomas. Long non-coding RNAs (lncRNAs) are a member of non-coding RNAs, regulating the expression of gene. Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is an onco-lncRNA that is overexpressed in several carcinomas including ATC. Evidence showed that MALAT1 has a crucial function in apoptosis, and cell cycle progression.

Objectives In order to take advantage of 3D cell culture system in cancer investigation, we have used a 3D in vitro ATC model to determine the effect of dual MEK/Aurora kinase inhibitor BI-847325 anticancer drug on the fundamental molecular mechanisms of MALAT1-mediated gene regulation in ATC.

Methods In this study, ATC cell lines (C643 and SW1736) were grown in alginate scaffold. Encapsulated cells were treated by BI-847325. Changes in expression of *MALAT1*, *Mcl1*, *miR-363-3p*, and *cyclinD1* were measured by qRT-PCR.

Results and conclusion *MALAT1* gene expression following BI-847325 treatment was significantly downregulated in C643 and SW1736 cell lines. Reversely, *miR-363-3p* expression was significantly upregulated by BI-847325 in both ATC cell lines. *Mcl1* expression was significantly downregulated after treatment in C643 cell lines. Moreover, the expression of this gene was not significantly reduced following BI-847325 treatment in SW1736 cell line. Additionally, *cyclin D1* expression was significantly downregulated after treatment in both ATC cell lines. Altogether, the result of this study was the first report of MALAT1's molecular function in ATC and suggested that BI-847325 which inhibits both MEK and Aurora kinase family could be effective against ATC by regulating the genes involved in cell cycle and apoptosis including MALAT1 and its downstream genes.

Keywords Anaplastic thyroid cancer · BI-847325 · MALAT1 · miR-363-3p · Mcl1 · Cyclin D1

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Introduction

Thyroid carcinoma is the most prevalent endocrine malignancy and has the highest increasing incidence rate between the other kinds of cancers in the United States [1]. Anaplastic thyroid carcinoma (ATC) is extremely aggressive and has a poor prognosis. Despite the fact that ATC is rare (2–5%), it is responsible for more than 50% of thyroid carcinoma associated mortality [2]. In most cases, ATC patients do not survive more than six months after diagnosis [3]. Current treatment approaches for ATC are not effective against the disease, perhaps because they do not adequately target the biological processes that are modulated by long non-coding RNAs (lncRNAs), as gene master regulators [4–6].

In recent years, investigators in the field of cancer biology have demonstrated that several major cellular processes are modulated by lncRNAs, including apoptosis, cell cycle, and metastasis [7]. lncRNAs are a category of non-coding RNAs longer than 200 nucleotides in length and in most cases do not encode proteins. These molecules are transcribed from thousands of loci in human genomes and play important roles in gene regulation [8]. Though the roles of the majority of lncRNAs have not presently been distinguished, current investigations have illustrated that they are involved in carcinogenesis [9, 10].

Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is one of the most expressed lncRNA that is longer than 8000 nucleotides in length. MALAT1 was first recognized as a prognostic biomarker in stage I non-small cell lung cancer patients [11, 12].

Currently, studies have demonstrated that MALAT1 is involved in tumorigenesis, apoptosis, and cell cycle progression in different kinds of carcinoma, including papillary thyroid carcinoma (PTC), gastric cancer, and cervical cancer [13, 14]. Consequently, MALAT1 is often considered as a biomarker for variety of cancers [15, 16]. Previous studies indicated that MALAT1 localizes in nuclear speckles, which are important elements in the splicing process [17]. Several studies showed that MALAT1 has key biological roles including the modulation of cell cycle and apoptosis in liver and gallbladder cancer cells [18]. Though, the biological function of lncRNA MALAT1 in ATC progression and its mechanisms of function concerning the modulation of cell cycle- and apoptosis-associated genes remain unclear.

It is now becoming clear that the MEK/ERK signaling pathway is involved in MALAT1 gene expression [19]. ERK2 is one of the members of the mitogen-activated protein kinase (MAPK) super family, which can modulate

cell cycle and apoptosis. The bioinformatics data indicated that ERK2 is positively correlated with lncRNA MALAT1. Recently, the experimental analysis showed that ERK2 is able to enhance cardiomyocytes proliferation by increasing lncRNA MALAT1 [18, 20]. In the present study, in order to take advantage of 3D cell culture system in cancer investigation, we have used the 3D in vitro ATC model to determine the effect of dual MEK/Aurora kinase inhibitor “BI-847325” anticancer drug on the fundamental molecular mechanisms of MALAT1-mediated gene regulation in ATC cell lines.

Methods

Materials

Human ATC C643 and SW1736 cell lines were purchased from CLS (Germany). BI-847325 was bought from Adooq Bioscience (Irvine, CA, USA). Cell culture medium and fetal bovine serum were obtained from Invitrogen (Carlsbad, CA, USA). Glutamine, penicillin, streptomycin, and sodium alginate were provided by Sigma Aldrich (Germany). Sodium citrate, CaCl_2 and, HEPES were purchased from Merck (Darmstadt, Germany). RNA extraction kit was obtained from Qiagen (Valencia, CA, USA). cDNA synthesis kit and SYBR green were provided by Fermentas (Waltham, Massachusetts, USA).

2D cell culture

ATC C643 and SW1736 cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units of penicillin/ml, and 100 mg of streptomycin/ml. Both cell lines were incubated in 37 °C with 5% CO_2 .

Alginate encapsulation

1.2% (w/v) sodium alginate was prepared in HEPES buffer (20 mM + NaCl 155 mM, pH 7.4). CaCl_2 solution (102 mM) was prepared using HEPES buffer. C643 and SW1736 cell lines were resuspended in alginate hydrogel at 5×10^5 viable cells/ml. The hydrogel -cell suspension was extruded through a 22-G needle into the CaCl_2 solution. The resultant alginate beads remained in the CaCl_2 solution for 10 min at room temperature. The alginate beads were consequently washed with PBS. Afterward, cells were cultured in beads for five days. C643 and SW1736 cell lines were treated with 15 μM and 34 μM

concentrations (based on our unpublished manuscript) of BI-847325 for 48 h before RNA isolation.

RNA isolation, cDNA synthesis, and q-RT PCR

Cells were revived from the beads using sodium citrate (55 mM, 10 mM HEPES, pH 7.4). Total RNA was extracted from cultured cell lines using Qiagen's RNeasy mini kit, conforming to the manufacturer's protocol. Total RNA was reverse-transcribed by revert aid first-strand cDNA synthesis kit conforming to the manufacturer's protocols. *miR-363-3p* was quantified conforming to stem-loop methodology, described previously by Naderi et al. [21]. Changes in *MALAT1*, *Mcl1*, and *cyclin D1* gene expression were measured by qRT-PCR with SYBR green. All reactions were accomplished in duplicate. Changes in miRNA and mRNA expression were normalized to *snord47* and β -actin, respectively. All the expression computations were accomplished by $2^{-\Delta\Delta C_t}$ method. Reactions were performed at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. All the primer pair sequences are listed in Table 1.

Statistical analysis

All the data show mean of three independent experiments $\pm$ SD. Statistical comparisons were analyzed by two-way

Table 1 The sequences of the primers

No.	Gene	Sequence
1	MALAT1	F: GTAACGATGGTGTGCGAGGTC R: CAGCATTACAGTTCTTGAACATG
2	Mcl1	F: AACAAAGAGGCTGGGATG R: ATTGCACTTACAGTAAGGCTATC
3	cyclin D1	F: CCCTCGGTGTCCTACTTCAAATG R: CCTCCTCGCACTTCTGTTC
4	miR-363-3p	Stem loop RT: GTCGTATGCAGTGCAGG GTCCGAGGTATTGCGACTGCATACGA CTACAGA F: AGGCAATTGCACGGTATCC R: GTGCAGGGTCCGAGGT
5	β -actin	F: CTCCTTCCTGGGCATG R: GTCTTTGCGGATGTCCAC
6	Snord47	Stem loop RT:GTCGTATGCAGAGCAGG GTCCGAGGTATTGCGACTGCATACG ACAACCTC F: ATC ACTGTAAAACCGTTCCA R: GAGCAGGGTCCGAGGT

ANOVA using GraphPad Prism software (GraphPad PRISM V 5.0). Statistical significances are expressed as $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***); $p < 0.0001$ (****). In addition, qRT-PCR analysis was performed using R packages gplots [22, 23].

Results

BI-847325 caused *MALAT1* downregulation in ATC cell lines

In this study, we examined *MALAT1* gene expression following BI-847325 treatment. The data illustrated that after treatment the expression of *MALAT1* in C643 ($p < 0.01$) and SW1736 ($p < 0.001$) cell lines was significantly decreased in comparison with untreated control (Figs. 1 and 2).

BI-847325 decreased *Mcl1* by BI-847325

Post BI-847325 treatment, the expression of *Mcl1* was evaluated to observe the apoptosis-associated gene induction in both cell lines. The expression of *Mcl1* was significantly ($p < 0.001$) downregulated after BI-847325 treatment in C643 cell lines. Furthermore, the expression of this gene was not significantly downregulated following treatment in SW1736 cell line (Figs. 1 and 2).

BI-847325 increased *miR-363-3p* in C643 and SW1736 cell lines

Our results indicated that the amount of *miR-363-3p* was significantly ($p < 0.0001$) elevated in BI-847325-treated cell lines in comparison with untreated control (Figs. 1 and 2).

BI-847325 modulated cell cycle-associated gene through downregulation of *cyclin D1*

Cyclin D1 gene expression was used as a cell cycle progression marker following BI-847325 treatment on both cell lines (Figs. 1 and 2). The expression of *cyclin D1* was significantly downregulated after BI-847325 treatment in ATC cell lines, C643 ($p < 0.01$) and SW1736 ($p < 0.001$). The differences between gene expression patterns of *MALAT1*, *Mcl1*, *miR-363-3p*, and *cyclin D1* in C643 and SW1736 cell lines are shown in Fig. 3.

Fig. 1 The expression of *MALAT1*, *Mcl1*, *miR-363-3p*, and *cyclin D1* genes in C643 cell line. **a** Gene expression of *MALAT1*, *Mcl1*, and *cyclin D1* was decreased in C643 cell line 48 h after BI-847325 treatment. *miR-363-3p* was upregulated in treated cells. All the expressions were compared to untreated control. The data are presented as the means of three independent experiments $\pm$ SD. Statistical significances are expressed as $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***); $p < 0.0001$ (****). **b** Gplot heatmap of gene expression data. Expression level is color coded: crimson for overexpressed, light blue for unchanged expression, and dark blue for underexpressed genes

Discussion

The results of this study indicate that BI-847325 can affect on cell cycle- and apoptosis-associated genes that previously reported to be involved in these processes by downregulating of *MALAT1* gene expression and its downstream effectors in ATC. In general, ATC cells are known to have high proliferation and low apoptosis [24, 25]. Regrettably, despite advances in cancer biology, the poor survival of ATC is one of the major challenges of cancer treatment era [2, 4, 26]. Therefore, there is an important need to develop more effective treatments against ATC according to the molecular mechanisms involved in cancer including lncRNAs.

In thyroid carcinoma, just a few of lncRNAs were discovered to be important in tumorigenesis and cancer progression.

Several studies have shown that activation of MEK/ERK signaling pathway is associated with elevated *MALAT1* gene expression [18–20]. In recent years, increasing number of papers have indicated that lncRNAs function as sponges to bind particular miRNAs and modulate their activity. In pancreatic adenocarcinoma, Gao and colleagues demonstrated that upregulation of lncRNA ROR is involved in the increase of Nanog by sponging of miR-145 [27]. Another report indicated that a new lncRNA, C032469, is upregulated in gastric cancer.

Fig. 2 The expression of *MALAT1*, *Mcl1*, *miR-363-3p*, and *cyclin D1* genes in SW1736 cell line. **a** Gene expression of *MALAT1*, and *cyclin D1* was decreased in SW1736 cell line 48 h after BI-847325 treatment. *miR-363-3p* was upregulated in treated cells. Moreover, the expression of *Mcl1* gene was not significantly reduced following BI-847325 treatment in SW1736 cell line. All the expressions were compared to untreated control. The data are presented as the means of three independent experiments $\pm$ SD. Statistical significances are expressed as $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***); $p < 0.0001$ (****). **b** Gplot heatmap of gene expression data. Expression level is color coded: crimson for overexpressed, light blue for unchanged expression, and dark blue for underexpressed genes

LncRNA C032469 could bind to a specific target miRNA, miR-1207-5p, and efficiently functions as a sponge for miR-1207-5p to regulate the human telomerase reverse transcriptase (hTERT) gene expression [28]. Similarly, MALAT1 functions as a miRNA sponge to decrease the endogenous role of miR-363-3p, which negatively regulates *Mcl1* expression in ATC (Graphical abstract). *miR-363-3p* expression was significantly upregulated by BI-847325 treatment in both ATC cell lines. Reversely, changes in *Mcl1* and *MALAT1* gene expression were significantly down-regulated by BI-847325 treatment in C643 cell line. Moreover, *Mcl1* expression was not significantly downregulated following treatment in the

SW1736 cell line, despite the fact that this gene is a target of MEK/ERK signaling pathway. In general, MALAT1 functioned as a miRNA sponge to decrease the endogenous role of miR-363-3p that adversely regulates *Mcl1* gene expression in ATC.

Li and et al. demonstrated that MALAT1 can activate Wnt/ β -catenin signaling pathway in hepatocytes. In these cells, Axin1 and APC are downregulated by MALAT1, and the stable β -catenin translocates from the cytosol to the nucleus, where it enhances *cyclin D1* transcription and promotes cell cycle progression from the G₁ to the S phase [29].

In this study, to investigate the effects of BI-847325 on cell cycle-associated gene in ATC, its effect on

Fig. 3 Gplot heatmap of gene expression data in C643 and SW1736 cell lines. The differences between gene expression patterns of *MALAT1*, *Mcl1*, *miR-363-3p*, and *cyclin D1* in C643 and SW1736 cell lines are shown in color coded: crimson for overexpressed, light blue for unchanged expression, and dark blue for underexpressed genes

cyclin D1 gene expression was evaluated. *Cyclin D1* is an oncogene as well as the cell cycle checkpoint regulator which causes cell cycle arrest in G₁ phase. *Cyclin D1* gene expression was significantly downregulated after BI-847325 treatment in ATC cell lines, C643 and SW1736 (Graphical abstract). In general, this study indicates that BI-847325 which inhibits both MEK and Aurora kinase family could be effective against ATC by regulating the genes involved in cell cycle and apoptosis including MALAT1 and its downstream genes such as *miR-363-3p*, *Mcl1*, *cyclin D1*.

Conclusion

In conclusion, the result of this study is the first report of MALAT1's molecular function in ATC cells and suggests that the expression of MALAT1-mediated cell cycle- and apoptosis-associated genes can be affected using BI-847325 on ATC. MALAT1 is expected to become a novel biomarker in ATC. In addition, these results suggest that targeting the lncRNA MALAT1 regulatory network may be a more effective therapeutic strategy for ATC treatment.

Moreover, using cellular techniques and tissue samples from ATC patients are required to confirm the role of lncRNA MALAT1 in ATC. These findings could be warranted by ATC animal models.

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Authors' contributions MS, the chief instructor of the experiment, and HS performed the experiments, interpreted the data, and wrote the first draft of the manuscript. Other authors contributed to the experimental procedures. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Abbreviations ATC, anaplastic thyroid carcinoma; EOC, epithelial ovarian cancer; EZH2, enhancer of zeste homolog; GFs, growth factors; hTERT, human telomerase reverse transcriptase; lncRNAs, long non-coding RNAs; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; MAPK, mitogen-activated protein kinase; miRNA, micro ribonucleic acid; MMP14, matrix metalloproteinase 14; NAMA, non-protein coding RNA associated with MAPK signaling pathway and growth

arrest; *PTC*, papillary thyroid carcinoma; *PVTI*, plasmacytoma variant translocation 1; *RTK*, receptor tyrosine kinase; *ROR*, regulator of reprogramming; *TSHR*, thyroid-stimulating hormone receptor; *UCA1*, urothelial carcinoma-associated 1

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