Experimental and Molecular Pathology 99 (2015) 492–497

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Multiple proliferation-survival signalling pathways are simultaneously active in *BRAF* V600E mutated thyroid carcinomas

Md Atiqur Rahman [a], Ali Salajegheh [a], Robert Anthony Smith [a,b], Alfred King-yin Lam [a,c,*]

[a] Cancer Molecular Pathology, School of Medicine, Menzies Health Institute Queensland, Griffith University, Gold Coast, Queensland, Australia
[b] Genomics Research Centre, Institute of Health and Biomedical Innovation, Faculty of Health, Queensland University of Technology, Brisbane, Queensland, Australia
[c] Pathology Queensland and Gold Coast University Hospital, Gold Coast, Queensland, Australia

ARTICLE INFO

Article history:
Received 28 August 2015
Accepted 18 September 2015
Available online 25 September 2015

Keywords:
Thyroid carcinoma
BRAF
cell signalling pathway
ERK
AKT

ABSTRACT

Background and objectives. *BRAF* is an oncogene which involves in pathogenesis of many thyroid carcinomas.The aim of our study was to investigate whether the downstream signalling pathway of BRAF and AKT kinase signalling pathways were active in *BRAF* V600E mutated thyroid carcinoma cells. *Methods.* Five thyroid (papillary and undifferentiated) carcinoma cell lines and one non-cancer thyroid cell line were screened for their *BRAF* V600E mutation status by immunofluorescent staining and Western blot. *BRAF* V600E mutated thyroid carcinoma cell lines were used to test the activation status of both ERK and AKT kinase proteins through immunofluorescent studies and Western blots. *Results.* Expressions of *BRAF* V600E mutated protein were confirmed in four thyroid (papillary and undifferentiated) carcinoma cell lines. In these cell lines, both active ERK and active AKT kinase proteins were found in *BRAF* V600E mutated thyroid carcinoma cells by immunofluorescent staining and Western blots experiments. *Conclusions.* In *BRAF* V600E mutated thyroid carcinomas, active ERK and active AKT kinase proteins were noted. They are able to stimulate multiple downstream signalling pathways which ultimately result in increased proliferation and survival activities for cancer cells. Therefore, consideration needs to put on multiple targets when deciding molecular target therapies for patients with *BRAF* V600E mutated thyroid carcinoma.

1. Introduction

Thyroid carcinoma is most frequent in endocrine malignancy and is more common in women. In addition, papillary thyroid carcinoma is most common among all thyroid carcinomas whose prevalence is more than 70% of all thyroid cancers (Lam et al., 2005). Undifferentiated thyroid carcinoma accounted for approximately 4% of thyroid carcinomas. It is the most aggressive variant of thyroid carcinoma and many can be proved to be derived from papillary thyroid carcinoma (Lam et al., 2000; Lo et al., 1999).

BRAF is an oncogene and oncogenic mutation in *BRAF* involves in pathogenesis of cancers such as melanoma, colorectal cancer, and ovarian serous tumours (Pakneshan et al., 2013). The overall prevalence of the mutation in human cancer is approximately 8% (Rahman et al., 2013). This type of mutation was first discovered in thyroid carcinoma in 2003 (Cohen et al., 2003). It is uniquely seen in papillary thyroid carcinoma and papillary thyroid carcinoma-derived undifferentiated thyroid carcinoma. The prevalence of this mutation is approximately 45% in papillary thyroid carcinoma and 24% in-papillary thyroid carcinoma derived undifferentiated thyroid carcinoma (Smith et al., 2011; Xing, 2005). Patients with *BRAF* mutated papillary thyroid carcinoma are associated with increased mortality and increased cancer recurrence (Xing et al., 2013; Xing et al., 2015). Thus, papillary and undifferentiated thyroid carcinomas harbouring *BRAF* mutation could be benefited from treatment with therapy targeted against *BRAF* mutated protein.

Small molecular kinase inhibitors are developed to target mutant BRAF protein in cancer. Currently, only a Phase I clinical trial report has been published and a Phase II clinical trial (ClinicalTrials.gov ID: NCT01286753) is ongoing on *BRAF* mutated thyroid carcinoma. So far, the maximum response rate of these BRAF inhibitors is noted not more than 50% in thyroid carcinoma or melanoma (Rahman et al., 2014a; Dadu et al., 2015; Kim et al., 2013). Moreover, patients undergoing BRAF inhibitor therapy develop resistance against BRAF inhibitor within few months of treatment initiation (Dadu et al., 2015; Kim et al., 2013; Rahman et al., 2014b). The reasons for BRAF inhibitor resistance had been investigated and found that most of the BRAF inhibitor resistance happened due to reactivation of ERK proliferation-survival kinase signalling pathway and a second activation of AKT proliferation-survival kinase signalling pathway (Rahman et al., 2014b).

It is not quite clear whether AKT proliferation-survival kinase signalling pathway is activated after BRAF inhibitor treatment or this kinase signalling pathway is already active in the first place in thyroid

* Corresponding author at: Griffith Medical School, Gold Coast Campus, Gold Coast, QLD 4222, Australia.
E-mail address: a.lam@griffith.edu.au (A.K. Lam).

http://dx.doi.org/10.1016/j.yexmp.2015.09.006
0014-4800/

carcinoma. There is no previous data available. Therefore, the aim of this study was to check activation status of both ERK and AKT proliferation-survival kinase signalling pathways in *BRAF* V600E mutated thyroid carcinoma which has not yet been investigated in *BRAF* V600E mutated thyroid carcinoma.

2. Materials and methods

2.1. Cell lines and culture

Thyroid carcinoma cell lines, B-CPAP (papillary thyroid carcinoma), 8505C (undifferentiated thyroid carcinoma), BHT-101 (undifferentiated thyroid carcinoma) and MB-1 (undifferentiated thyroid carcinoma) were procured from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmBH — German collection of microorganisms and cell cultures (DSMZ).

In addition, papillary thyroid carcinoma cell line (K1) and non-cancer thyroid epithelial cell line (Nthy-ori 3-1) were obtained from the European Collection of Cell Cultures (ECACC). All the cell lines were authenticated by short tandem repeat (STR) profiling which was accomplished by the supplier company before purchased. Earlier passaged (passage number less than five) cell lines were used for every experiments. In addition, cell morphology of all the cell lines was checked by the author (AKL, a pathologist) before each experiment to confirm the diagnosis. Company suggested protocol was followed and recommended medium was used for culturing and preservation of each cell line. 8505C, B-CPAP and Nthy-ori 3-1 cells were cultured with Roswell Park Memorial Institute medium-1640 (RPMI-1640) (Invitrogen, Carlsbad, CA, USA)supplemented with 10% foetal bovine serum (Invitrogen) and 2 mM L-glutamine (Invitrogen). BHT-101 cells were cultured with Dulbecco's Modified Eagle's medium (DMEM) (Invitrogen) supplemented with 20% heat & inactivated foetal bovine serum (Invitrogen), 0.5% human serum (Sigma-Aldrich, St. Louis, MO, USA) and 5 μg/ml human insulin (Sigma-Aldrich). MB-1 cells were cultured with RPMI-1640 (Invitrogen) medium supplemented with 20% heat & inactivated foetal bovine serum (Invitrogen) and 2 mM L-glutamine (Invitrogen). K1 cells were cultured with DMEM (Invitrogen), Ham's F12 (Invitrogen) and MCDB 105 (Sigma-Aldrich) medium in a ratio 2:1:1 supplemented with 10% heat & inactivated foetal bovine serum (Invitrogen) and 2 mM L-glutamine (Invitrogen). Culture medium was also supplemented with antibiotics 1X penicillin–streptomycin (Invitrogen).

2.2. Immunofluorescent

3×10^4 cells were seeded on coverslip in every well of the 24 wells plate containing complete culture medium supplemented with serum and antibiotics. After 80% confluence, cells on coverslip were fixed with cold 4% paraformaldehyde (pH 7.2–7.3) incubated for 15 min at room temperature followed by permeabilization with 0.25% Triton X-100 incubated for 15 min at room temperature and background blocking with 5% goat or rabbit serum incubated for 60 min at room temperature. Then, cells on coverslip were incubated with primary antibody overnight at 4 °C. On the following day, cells on coverslip were incubated with Alexa Fluor conjugated secondary antibody for 1.5 h at room temperature in a dark room. After that, cells on coverslip were counterstained with DAPI (4′, 6-diamidino-2-phenylindole) incubated for 5 min in dark and the coverslip was attached on glass slide. Images were captured with Nikon A1R + Confocal microscope (Nikon, Melville, NY, USA) and the image acquisition software was NIS Elements Confocal Version 4.13 (Nikon). Antibodies and dilutions used were: BRAF V600E (1:100), BRAF (1:100), p-ERK (1:100), p-AKT (Ser 473) (1:100), rabbit anti-goat IgG (Alexa Fluor 594) (1:1000), donkey anti-rabbit IgG (Alexa Fluor 594) (1:1000) and goat anti-mouse IgG (Alexa Fluor 488) (1:1000). BRAF, p-ERK and p-AKT antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). BRAF V600E antibody was purchased from Spring Bioscience (Pleasanton, CA, USA). Alexa Fluors and DAPI were purchased from Life Technologies (Carlsbad, CA, USA).

2.3. Western blots

In a 10 cm diameter cell culture disk, 5×10^5 cells were seeded in 10 ml of complete culture medium containing serum and antibiotics. Total cell lysates were extracted from cells after 80% confluence by using NP40 Cell Lysis Buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1% Nonidet P40, 0.02% NaN_3) procured from Invitrogen. The manufacturer's recommended protocol was followed during cell lysate extraction. NP40 Cell Lysis Buffer was supplemented with protease inhibitor cocktail (Sigma, St. Louis, MO, USA), phenylmethanesulfonyl fluoride solution (PMSF) (Sigma-Aldrich) and phosphatase inhibitor cocktail (Cell Signaling, Danvers, MA, USA).

30 μg total cell lysate was resolved through 4–15% gradient SDS-PAGE gel (BIO-RAD, Hercules, CA, USA) and transferred to polyvinylidene fluoride membrane (BIO-RAD). Subsequently, the membrane was blocked with 5% non-fat milk for detecting total protein or with 5% bovine serum albumin (BSA) for detecting phosphorylated protein incubating for one hour. The blocked membrane was then incubated overnight with primary antibody at 4 °C followed by two hours incubation with HRP (Horseradish peroxidase) conjugated secondary IgG (Santa Cruz Biotechnology). Eventually, blots were developed with Immun-Star WesternC chemiluminescence kit (BIO-RAD) and visualized by using ChemiDoc MP Imaging System (BIO-RAD). The results were analysed with Image Lab software version 5.2.1 (BIO-RAD). Antibodies and dilutions used were: BRAF V600E (1:500), BRAF (1:500), p-ERK (1:300), ERK (1:300), p-AKT (Ser 473) (1:400), AKT (1:500), GAPDH (1:1000), goat anti-rabbit IgG-HRP (Horseradish peroxidase) (1:5000), goat anti-mouse IgG-HRP (1:5000) and rabbit anti- goat IgG-HRP (1:5000). BRAF V600E antibody was purchased from Spring Bioscience. All other antibodies were purchased from Santa Cruz Biotechnology.

2.4. Statistical analyses

Experimental results were presented as mean values ± standard error of the mean (SEM). All statistical analyses were accomplished with GraphPad Prism 6 software (GraphPad Software Inc., San Diego, California, USA).

3. Results

3.1. 8505C, B-CPAP, K1 and BHT-101 thyroid carcinoma cell lines were BRAF V600E mutated

Immunofluorescent experiment was done to confirm the *BRAF* V600E mutational status of thyroid carcinoma cell lines (8505C, B-CPAP, K1, BHT-101, MB-1) and non-cancer thyroid control cell line (Nthy-ori 3-1) using BRAF V600E specific antibody to detect BRAF V600E mutated protein expressed in cells. Positive signals were noted in 8505C, B-CPAP, K1 and BHT-101 thyroid carcinoma cell lines (Fig. 1). MB-1 and Nthy-ori 3-1 thyroid cell lines did not expressed BRAF V600E mutated protein, although these two cell lines expressed BRAF protein (Fig. 1).

Western blot experiment also revealed that BRAF V600E mutated proteins were only expressed in four thyroid carcinoma cell lines (8505C, B-CPAP, K1 and BHT-101) (Fig. 2A). On the other hand, BRAF V600E mutated proteins were not expressed in MB-1 and non-cancer (Nthy-ori 3-1) thyroid cells, though BRAF proteins were expressed in these two types of cells (Fig. 2A). Furthermore, blots analysed with Image Lab software showed that 8505C and B-CPAP thyroid carcinoma cells expressed almost double amount of BRAF V600E mutated proteins when compared to K1 and BHT-101 thyroid carcinoma cells (Fig. 2B).