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Original contribution

Enhanced B-Raf protein expression is independent of V600E mutant status in thyroid carcinomas☆

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Summary *BRAF* (7q24) encodes a serine/threonine protein kinase, and its expression level varies in different tissues. Although a high prevalence of *BRAF* mutation has been suggested as an important event in thyroid tumorigenesis, little is known about the expression pattern of B-Raf in the thyroid. Thus, we examined the expression of B-Raf in various neoplastic and nonneoplastic thyroid tissues and compared it with *BRAF* mutational status. Normal and hyperplastic thyroid tissues showed focal and faint immunoreactivity for B-Raf, especially in cuboidal follicular cells of small follicles. In contrast, diffuse expression of B-Raf was observed in follicular adenomas and well-differentiated carcinomas. The missense point mutation $BRAF^{V600E}$ was identified in 42% (13/31 cases) of papillary carcinomas and 33% (5/15 cases) of undifferentiated carcinomas but not in normal thyroid tissues, nodular hyperplasia, follicular adenomas, or follicular carcinomas. The immunohistochemical expression of B-Raf did not correlate with *BRAF* mutational status. Moreover, Western blotting revealed that B-Raf expression in thyroid carcinoma cell lines was also independent of *BRAF* mutation. Serum or fibroblast growth factor-1 stimulation further activates ERK1/2 in heterozygous $BRAF^{V600E}$-positive carcinoma cells as well as $BRAF^{V600E}$ mutation–negative carcinoma cells. In conclusion, heterogeneous focal expression of wild-type B-Raf in nonneoplastic tissues may play a role in the growth or functional activity of thyroid follicular cells. In contrast, diffuse expression of wild-type and/or mutant B-Raf may be involved in the tumorigenic process resulting in activation of the mitogen-activated protein kinase signaling pathway in cooperation with other genetic abnormalities and activation of ligand-receptor signaling pathways.

1. Introduction

Thyroid cancer is the most common malignancy in endocrine organs, and its incidence rates have steadily increased over the last decades [1]. More than 95% of thyroid carcinomas are derived from follicular cells, with a spectrum of differentiation from the most indolent carcinomas

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(papillary microcarcinoma) to the most invasive and lethal human malignancies (undifferentiated thyroid carcinoma) [1]. This spectrum of progression has been closely linked with a pattern of cumulative genetic defects that correlate with tumor differentiation, metastatic potential, and aggressiveness [1]. Of these, *RET* rearrangements (*RET*/PTC) and activating somatic mutations in *RAS* and *BRAF* oncogenes are mutually exclusive and represent important genetic events in thyroid carcinogenesis [2-5].

The proto-oncogene *BRAF*, situated on 7q24, encodes a serine/threonine kinase that transduces regulatory signals through the Ras/Raf/MEK/ERK cascade. B-Raf exhibits restricted expression in normal tissues and is highly expressed in neuronal tissues, testis, and hematopoietic cells [6]. Mice deficient of *BRAF* exhibit defects involving neuroepithelial differentiation and endothelial maturation [6]. Conversely, gain-of-function germline mutation of *BRAF* is associated with the cardiofaciocutaneous syndrome, with characteristic craniofacial features, cardiac defects, developmental delay, cutaneous hyperkeratosis, and multiple cavernous hemangiomata [7]. These observations suggest specialized expression and functions of B-Raf in normal tissues. On the other hand, somatic mutation of *BRAF* provides an alternative mode of aberrant activation of mitogen-activated protein kinase (MAPK) signaling. This mutation has been implicated in tumorigenesis of several human cancers, including thyroid carcinomas [4,5,8]. The most common *BRAF* point mutation in sporadic papillary thyroid carcinomas is a thymine-to-adenine transversion at nucleotide 1799 in exon 15 (T1799A), resulting in a valine–to–glutamic acid substitution at amino acid residue 600 ($BRAF^{V600E}$, formerly described as $BRAF^{V599E}$) [4,5].

The aim of the present study was to investigate the expression pattern of B-Raf protein and its mutational status in various thyroid tissues and thyroid carcinoma cell lines. In addition, we examined whether B-Raf expression levels can be correlated with histopathologic diagnosis of thyroid tumors.

2. Materials and methods

2.1. Case selection

We studied 127 surgical specimens, including normal thyroids (22 cases), Graves disease (8 cases), nodular hyperplasia (16 cases), follicular adenoma (19 cases), follicular carcinoma (16 cases), papillary carcinoma (31 cases), and undifferentiated carcinoma (15 cases) to represent a range of thyroid pathologies from routine surgical pathology files at University of Yamanashi Hospital, Yamanashi, Japan. Hematoxylin-eosin–stained slides of all cases were reviewed, and the diagnosis was made on the basis of the World Health Organization classification [9]. All papillary carcinomas were sporadic cases; age at diagnosis ranged from 14 to 83 years (median, 61 years), including 28 cases of conventional type and 3 cases of follicular variant. In current study, 9 in 15 cases of undifferentiated carcinomas were associated with papillary carcinoma component. Normal thyroid tissues were obtained from patients who underwent subtotal or total thyroidectomy for papillary carcinoma. Protocols were approved by the institutional ethics board of the University of Yamanashi.

2.2. Cell lines and cell culture

Human thyroid carcinoma-derived cell line, WRO (Dr J Fagin, University of Cincinnati, Cincinnati, OH; established by Dr G Juillard, University of California, Los Angeles, CA), TPC-1 (kindly provided by Dr S Yamashita, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan), KTC-1 [10], 8505C, and 8305C (provided by Cell Resource Center for Biomedical Research, Tohoku University, Sendai, Japan) were maintained in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% FBS, streptomycin sulfate (100 mg/L), and penicillin G sodium (100 mg/L). Cells were cultured in a standard humidified incubator at 37°C in a 5% carbon dioxide atmosphere. For determination of ERK1/2 phosphorylation status and protein expression, cultured cells were treated with fibroblast growth factor (FGF)-1 (25 ng/mL; Sigma, St Louis, MO) and 10 U/mL of heparin (Sigma) in serum-free medium or 10% FBS in medium after 24 hours of serum starvation. The identical volume of vehicle was also applied as a control for each experiment.

2.3. Immunohistochemistry and immunocytochemistry

Immunohistochemical analysis was performed on 3-μm sections of formalin-fixed and paraffin-embedded tissues. Detail protocol was as previously described [11]. Briefly, deparaffinized sections were incubated with a primary antibody at room temperature for 2 hours. Primary antibodies and working dilution rates were as follows: B-Raf, 1:50 dilution (sc-9002, polyclonal antiserum, Santa Cruz Biotechnology, Santa Cruz, CA) and ERK1, 1:50 (sc-93, polyclonal antiserum, Santa Cruz Biotechnology). Both antibodies were diluted with 1% bovine serum albumin–phosphate-buffered saline (PBS). To visualize the reaction, the labeled polymer method (Envision/horseradish peroxidase, DAKO, Glostrup, Denmark) was carried out according to the manufacturer's instructions. The immunoreactivity of B-Raf was evaluated using a scale from grades 0 through 3+: grade 0, no staining; 1+, focal (1%-9%); 2+, intermediate between 1+ and 3+; 3+, diffuse (>50%).

Indirect immunofluorescence staining was carried out in thyroid carcinoma cell lines. Cells were plated on Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL). After 24 hours' incubation with growth medium containing