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Original Article

Up-regulation of urinary-type plasminogen activator correlates with high-risk papillary thyroid carcinoma with BRAF^{V600E} mutation and its possible molecular mechanism

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ABSTRACT

The aim of the present study is to investigate the relationship between urinary-type plasminogen activator (uPA) expression and clinicopathological features in papillary thyroid carcinoma (PTC) and to determine the signal transduction of PTC cells *in vitro.*

PTC tissues from 42 patients were analyzed for the expression of uPA and the BRAF^{V600E} mutation. BCPAP, a PTC cell line harboring the BRAF^{V600E} mutation, was used to study MAPK signaling. PCR and direct sequencing were applied to analyze BRAF^{V600E} mutation status. uPA mRNA expression was measured using a quantitative RT-PCR method, and uPA protein was localized using an immunohistochemical method. The ERK protein status was detected by Western blot analysis.

uPA gene expression was significantly increased in PTC tissues as compared to the corresponding non-tumor tissues. Furthermore, the up-regulation of uPA mRNAs was correlated with high-risk clinicopathological features, including extrathyroid invasion, loss of cellular polarity/cohesiveness, and the BRAF^{V600E} mutation. Marked dephosphorylation of ERK1/2 and down-regulation of uPA expression were detected when BCPAP was treated with a MEK inhibitor, U0126.

MEK inhibitors might be a potential treatment strategy for aggressive PTC with BRAF^{V600E} through inhibition of uPA expression.

Introduction

Papillary thyroid carcinoma (PTC) is the most common malignant tumor of the human endocrine system. Although PTC is relatively indolent and highly curable, up to 10% of patients with PTC develop cancer recurrence and eventually die from this disease [10]. There are several clinicopathological features that are recognized as classical high-risk factors, including large tumor size, extrathyroid invasion, lymph node metastasis, distant metastasis and advanced tumor stages [6]. For PTC, histological subtype is also an important factor in the risk evaluation of this cancer. Recently, loss of cellular polarity/cohesiveness (LOP/C) in PTC, particularly in the invasive front, was proposed by Kakudo and colleagues to describe a unique subgroup of PTC, in which the cancer cells display a hobnail-like pattern or loose arrangement without well-formed papillary or follicular structures. A subsequent study from the same group demonstrated that this subtype can predict a high-risk for PTC recurrence [2]. Furthermore, PTC is associated with non-overlapping activating mutations of RET, NTRK, RAS, and BRAF [1,22]. Among these genetic defects, the BRAF^{V600E} mutation is the most common oncogene identified in sporadic PTC. Although

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there is still controversy, many studies have demonstrated that $BRAF^{V600E}$ mutation is correlated with the high-risk factors listed above and may be a driving force behind the aggressive clinicopathological characteristics of PTC [14].

On the other hand, in the complex tumor progression, a key requirement is the ability of tumor cells to produce proteolytic enzymes within the tumor cell environment to promote extracellular matrix degeneration to facilitate tumor invasion and metastasis [7]. One such protease, urinary-type plasminogen activator (uPA), has strongly been implicated in the progression of several malignancy, including thyroid cancer [8,11,21]. However, the correlation between increased expression of uPA and the high-risk clinicopathological features with genetic alterations of PTC is still poorly understood. Furthermore, *in vitro* studies suggest a significant correlation between uPA up-regulation and constitutive activation of the RAS-RAF-MEK-ERK/MAPK pathway (referred to as the MAPK pathway) in breast cancer [17]. All these observations have led us to hypothesize that $BRAF^{V600E}$ mutation could drive up-regulation of uPA through constitutive activation of the MAPK pathway and participate in the progression of high-risk group of PTC.

In the present study, we examined the expression difference of uPA in clinical samples of PTC and the corresponding non-neoplastic tissues and evaluated its clinical correlation. We then used a PTC cell line with $BRAF^{V600E}$ mutation to investigate uPA expression and its changes upon suppression of the MAPK pathway with a MEK-1 inhibitor.

Materials and methods

Patients and tissue samples

Matched tumor tissues and non-neoplastic tissues were collected from 42 patients (mean age: 54.7 ± 17.0 years, 7 men and 35 women) with primary PTC who underwent thyroidectomy at Kuma Hospital (Kobe, Japan). All patients gave their written informed consent to the ethics committee of Kuma Hospital. None of the 42 patients had distant metastasis at the time of surgery.

After surgical resection, each thyroid tissue was handled in two ways: one part of thyroid tissue was snap-frozen immediately and stored at $-80\,^{\circ}C$ for RNA extraction; the remnants were fixed in formalin and embedded in paraffin for histopathological examination. Serial sections were cut from paraffin blocks and prepared for hematoxylin and eosin (HE) staining and immunohistochemistry.

Histological evaluation and immunohistochemistry

All HE sections were reviewed by three pathologists (Y.B., Y.L. and K.K.) to confirm the histological diagnosis according to the WHO classification of thyroid tumors (2004). Only common-type PTCs were included in the current study, and aggressive variants, such as tall cell, columnar cell, and solid variants, were excluded [6]. Loss of cellular polarity/cohesiveness (LOP/C) in the invasive front was evaluated according to the criteria previously proposed by our group [3,13,16]. Immunohistochemistry was performed using human anti-uPA antibody (No. 3689, 1:25 dilution, American Diagnostica, CT).

Cell culture

The human PTC-derived BCPAP cell line, which harbors the $BRAF^{V600E}$ mutation, was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany); the other PTC-derived TPC-1 cell line with RET/PTC-1 rearrangement was provided by Dr. R. Katoh (University of Yamanashi, Japan). These cell lines were routinely maintained in RPMI1640 medium (Invitrogen, Japan) supplemented with 10% fetal bovine serum (FBS) (HyClone, UT) in 5% CO_2 at $37\,^{\circ}C$. For *in vitro* experiments, 80% confluent cells were cultured in 0.5% serum-containing medium for 18 h and then treated with or without 10 μmol/L U0126 (Calbiochem, CA) for different time periods before extraction of RNA and protein [15,17].

Total RNA isolation, RT-PCR, and detection of BRAF mutation

Total RNA was isolated from each cell line and tissue samples using the ULTRASPEC RNA Isolation System (Biotecx, TX) [18]. cDNA was synthesized using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, CA). The PCR reaction primers for uPA and BRAF were as follows: uPA forward 5′-AAGGACTACAGCGCTGACAC-3′, reverse 5′-AACTCCTGCAGGCTTCAGTC-3′; BRAF forward 5′-GCACAGGGCATGGATTACTT-3′, reverse 5′-GATGACTTCTGGTGCCATCC-3′. RT-PCR analysis with β-actin primers was used as an internal quality control [18]. In addition, the purified PCR products of cDNA for the BRAF gene (exon 15) were sequenced using PCR primers as previously described [15].

Quantitative real-time PCR

Quantitative real-time PCR was carried out in an ABI PRISM 7000 sequence detection system apparatus (Applied Biosystems, CA) according to Taqman Protocol. Primer and probe sets used for analysis of uPA (Hs00170182_m1) and the endogenous reference, β-actin (Catalog #4310881E) transcripts were purchased from Applied Biosystems (Applied Biosystems, CA). The PCR amplification was performed in a final volume of 20 μl containing: TaqMan Gene Expression Assay (uPA), cDNA template and Taqman Universal PCR Master Mix. Each sample was measured in triplicate, and the expression level of the tested gene was normalized to the housekeeping gene (β-actin). Differences in gene expression between the PTC tissues and the corresponding non-neoplastic tissues were calculated using the formula $2^{\exp}(\Delta C_{t\,\text{tumor}} - \Delta C_{t\,\text{normal}})$ as previously described [3]. The results are expressed as fold changes.

Western blot analysis and ELISA assay

Thirty micrograms of total protein from each sample was analyzed by 10% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes (Millipore, MA). Membranes were incubated with anti-ERK antibody (Cell signaling, MA; #9102, 1:1000) or anti-pERK antibody (Cell signaling, MA; #9101, 1:1000) overnight at $4\,^{\circ}C$ [18]. This was followed by incubation with HRP-coupled anti-rabbit IgG secondary antibody (Dako, Denmark; 1:2000) at room temperature for 1 h, and the signals were detected with an enhanced chemiluminescence (ECL) kit (Amersham, UK). Three independent experiments were performed.

For determining the secreted uPA levels in the medium of cultured cells, ELISA assay was also performed. Briefly, BCPAP cells were pre-cultured in 0.5% serum-containing medium for 18 h, plated at 5×10^4 cells in 60-mm dishes, and treated with or without 10 μmol/L U0126. After incubation for different time-periods, the medium was collected. uPA ELISA kits (American Diagnostica, CT) were used to measure the concentrations of the uPA in cell culture supernatants. The data were expressed as the mean ± SE of the determinations from three independent experiments.

Statistical analysis

The comparison of uPA mRNA expression levels between tumor tissues and matched non-neoplastic tissues was performed using paired *t*-test. The Correlation between uPA mRNA expression and clinicopathological variables was analyzed statistically using