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Global profiling of the signaling network of papillary thyroid carcinoma

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ABSTRACT

Aims: Thyroid carcinoma is one of the most fast rising cancer diagnoses in the US. Papillary thyroid carcinoma (PTC) comprises 80% of thyroid carcinoma. The goal of our study is to identify regulatory proteins and signaling pathways altered in PTC.
Main methods: Protein Pathway Array (PPA) was applied to screen 65 signaling proteins and phosphoproteins in 27 pairs of PTC and surrounding benign tissues. Ingenuity Pathway Analysis (IPA) was applied to analyze the signaling pathway.
Key Findings: 11 were differentially expressed between tumors and surrounding tissues, 8 of which were up-regulated (cytokeratin 18, Stat 1, HMG-1, p-p70 S6 kinase, Raf-B, glutamine synthetase, p-PKC δ, and HDAC1), while 3 of which were down-regulated (cytokeratin 5, BECN1, and p-ERK). Further study showed that two proteins (p-p70 S6 kinase and cytokeratin 18) were associated with lymph node metastasis. The top 10 canonical pathways in PTC were identified to be involved in PTC.
Significance: Taken together, there is a broad array of dysregulation of signaling proteins in PTC, suggesting a heterogeneous group of diseases.

1. Introduction

Thyroid carcinoma is the third fast rising cancer diagnosis in the US [1]. In the last decade, the US Surveillance indicates an average annual percentage change of thyroid cancer of +5.2 in men and +5.7 in women. The US data reflect what occurred in most countries from both a quantitative and a qualitative standpoint. Papillary thyroid carcinoma (PTC) comprises 80% of thyroid carcinoma. Although the traditional pathologic examination through routine hematoxylin and eosin (HE) staining was the 'gold standard' diagnosis of PTC, some cases of PTC, particularly the follicular variant, show wide interobservable variability even among expert thyroid pathologists. Also, widespread use of ultrasonography and fine-needle aspiration biopsy may exemplify the epidemiologic term 'overdiagnosis' [2]. Therefore, the development of molecular biomarkers is important for both diagnosis and predicting progression via understanding molecular signaling pathways in PTC carcinogenesis.

Recent integrated genomics studies showed many genes and proteins are associated with carcinogenesis of PTC including mutations (BRAF, RAS, and RET/PTC) associated with aggressiveness (metastasis and recurrence), membrane receptors (MET and DORA1) involving in function of cell motility and proliferation, glycoproteins (FN1 and CDH16) associated with cell adhesion, and proteins (BCL2 and ITPR1) associated with apoptosis [3]. Integrated genomics analysis also revealed that many of the signaling pathways were linked to PTC, including the Wnt, mTOR, JAK–STAT, ErbB, MAPK, VEGF, and PPAR signaling pathway [4,5]. While these studies and others have markedly advanced our genomic understanding of PTC, the proteomic landscape of PTC has not been fully explored.

To understand the global alterations in signaling protein expression and activation, we investigated the expression of 65 proteins and phosphoproteins in MIBC tissues by means of Protein Pathway Array (PPA) method. These proteins and phosphoproteins were included in PPA in this study since they are part of functionally important signaling pathways involving in cell proliferation, apoptosis, invasion, and metastasis. PPA is a recently developed high-throughput protein assay, which combines multiplex immunoblotting with computational analysis. PPA can characterize hundreds of proteins in tissue samples and identify alterations in protein expression. Through PPA, we successfully found that 11 proteins showed a differential expression that of significance which is involved in transduction and activation of signaling pathways, regulating apoptosis, metabolism, cell proliferation, and progression.

2. Material and methods

2.1. Patients and tissue samples

A total of 27 patients were included in this study. The patient selection criteria were 1) histology diagnosis of papillary thyroid carcinoma,

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2) no treatment before surgery, and 3) sufficient tumor tissue for this study. All patients undergone thyroidectomy surgery in The First Affiliated Hospital of Sun-Yet Sen University. The clinicopathological data of the patients are summarized in Table 1. The TNM stage was done according to the American Joint Committee on Cancer (AJCC) [6]. This study was reviewed and approved by The First Affiliated Hospital of Sun-Yet Sen University's Institution Ethical Review Boards. Twenty-seven pairs of papillary thyroid carcinoma and surrounding benign tissues were obtained. The tumor and surrounding benign tissues were dissected and frozen within 30 min of removal in a liquid nitrogen tank after immediate pathological examination. Tumor samples of 3 × 3 × 5 mm were taken from areas without gross necrosis. Surrounding benign samples of 3 × 3 × 5 mm were taken from the same patient at 2 cm away from the tumor margin. The tumor samples did not contain normal mucosal tissue [7].

2.2. Protein Pathway Array

Proteins were extracted from tumors and surrounding benign tissues by a start process of lysis using 1 × sample lysis buffer (Cell Signaling Technology, Danvers, MA) containing 20 mmol/L Tris–HCL (pH 7.5), 150 mmol/L NaCL, 1 mmol/L Na2EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na3VO4, and 1 μg/mL leupeptin in the presence of 1 × proteinase inhibitor cocktail (Roche Applied Science, Indianapolis, IN) and 1 × phosphatase inhibitor cocktail (Roche Applied Science). Subsequently, the lysate was homogenized with a tissue grinder for 15 s and by sonication 3 times for 15 s each, and then centrifuged at14,000 rpm for 30 min at 4 °C. Theprotein concentrations were measured using BSA (BSA Protein Assay Kit from Pierce, Rockford, IL) method and adjust the final concentrations to 1 ~ 3 μg/μl using 1 × sample lysis buffer. Then approximately 300 μg of each protein sample was loaded and electrophoresed with prepared 10% SDS-PAGE gel containing 1.5 M Tris–HCL (PH8.8), 20% SDS, 40% acrylamide (29:1), 10% APS, and TEMED [8,9]. After electrophoresis, the proteins were transferred electrophoretically to a nitrocellulose membrane (Bio-Rad, Hercules, CA), which was subsequently blocked for 1 h with blocking buffer including either 5% milk or 3% bovine serum albumin in 1 × Tris–HCl, NaCl, and Tween 20 (TBST) containing 20 mmol/L Tris–HCl (pH 7.5), 100 mmol/L NaCl, and 0.1% Tween-20. Then, the membrane was clamped onto a Western blotting manifold (Mini-PROTEAN II Multiscreen Apparatus, Bio-Rad, Hercules, CA) that isolates 20 channels across the membrane. The multiplex immunoblot was performed using a total of 65 protein-specific or phosphorylation site-specific antibodies. A mixture of one to two antibodies in the blocking buffer was added to each channel and incubated at 4 °C overnight thereafter. Then the membrane was washed with 1 × TBS and 1 × TBST, and further incubated with secondary anti-rabbit or anti-mouse antibody conjugated with HRP (Bio-Rad) for 1 h at room temperature. The membrane was developed with chemiluminescence substrate (Immun-Star HRP Peroxide Buffer/Immun-Star HRP LuminolEnhancer, Bio-Rad) and chemiluminescent signals were captured using the ChemiDoc XRS System (Bio-Rad). Afterward, the same membrane was stripped using stripping buffer (Restore Western blot stripping buffer, Thermo Scientific, Rockford, IL) and then used to detect a second set of primary antibodies as mentioned above. The signals of each protein were determined by densitometric scanning (Quantity One software package, Bio-Rad). Differences in protein levels were determined by measuring the density of each band and normalized using internal standards.

Among 65 antibodies tested, 25 were specific for phosphorylation, including p-P44/42 MAPK (Erk1/2)(Thr202/Tyr204), p–p70 S6 kinase (Thr389), p-PKCδ (Thr505), p-PKCα (Ser657), p-PDK1 (Ser241), p-PKC α/βII (Thr638/641), p-PTEN (Ser380), p-STAT3 (Ser727), p-p38 (Thr180/Tyr182), p-GSK-3α/β (Ser21/9), p-p53 (Ser392), p-Akt (Ser473), p-Rb (Ser780), p-beta-catenin (Ser33/37/Thr41), p-c-Jun. (Ser73), p-EIF4B (Ser422), p-Met (Tyr1234), p-smad 1/5 (Ser463/465), p-ERK5 (Thr218/Tyr220), p-p90RSK (Ser380), p-CREB (Ser133), p-FAK (Tyr397), p-CDC2 (Tyr15), p-STAT5 (Tyr694), and p-RB (Ser807/811). Thirty antibodies were specific for signal transduction, including cytokeratin 5, BECN1, Stat1, HDAC1, Raf-B, cytokeratin 18, glutaminesynthetase, HMG-1, FAS, FOXM1, ERa, cytokeratin 19, Pax-2, keratin 10, HES1, FTα, IL-3Rα, Wnt-1, SYK, MetRS, twist, Lyn, KLF6, CaMKKa, SK3, E2A, ASCL1, JAK2, Jagged1, Arora A/AIK, FAH, FKHR, Axin, HCAM, Flt-3/Flk-2, PERK, CD33, uPA, NFATC1, and TCF1. All phosphorylation state-specific antibodies were obtained from Cell Signaling Technology (Danvers, MA), except for p-Met (Tyr1234), which was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and p-PKCα (Ser657), which was purchased from EMD Millpore (Billerica, MA). All non-phospho-antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), except for the following antibodies: 1) Stat1 and Arora A/AIK from Cell Signaling Technology (Danvers, MA); 2) glutamine synthetase from BD Biosciences (San Jose, CA); 3) cytokeratin 18 from DAKO (Carpinteria, CA); 4) FAH from Proteintech Group (Chicago, IL); and 5) keratin 10 from Covance (Princeton, NJ).

Table 1
Patient demographics and papillary thyroid carcinoma characteristics.

Clinicopathological characteristics	All patients, number N = 27 (%)
Age	
<45	15 (56)
≥45	12 (44)
Gender	
Male	6 (22)
Female	21 (78)
AJCC TNM stage[*]	
I	22(81)
II	3(11)
III	2(8)
T stage	
T1	21 (78)
T1a	6 (22)
T1b	15 (56)
T2	5 (19)
T3	1 (3)
Node status	
N0	22 (81)
N1	5 (19)
N1a	4 (16)
N1b	1 (3)

[*] According to the American Joint Committee on Cancer (AJCC).

2.3. Signaling network analysis

Cell signaling networks were created using Ingenuity Pathway Analysis (IPA, Version 9.0) (http://www.ingenuity.com). Differentially expressed proteins identified by Protein Pathway Array were imported into IPA and mapped to their corresponding gene objects in the Ingenuity Pathways Knowledge Base. Proteins were categorized on the basis of their molecular functions by using the software, mapped onto genetic networks in the IPA database, and then ranked by score (i.e., negative log of a p-value). The score is indicative of the proportion of proteins identified as differentially expressed in our analysis among all the molecules belonging to a particular network. In canonical pathways analyses, the most significant canonical pathways were identified and the value assigned to the biological functions was presented in -log (p-value) by the IPA tool.

2.4. Statistical analysis

Paired Student's t-test and Significant Analysis of Microarray (SAM) tool (http://www.stat.stanford.edu/~tibs/SAM) were used to select the proteins differentially expressed between tumors and normal tissues.