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Activation Status of Receptor Tyrosine Kinases as an Early Predictive Marker of Response to Chemotherapy in Osteosarcoma

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Abstract

Receptor tyrosine kinases (RTKs) are membrane receptors that play a vital role in various biological processes, in particular, cell survival, cell proliferation, and cell differentiation. These cellular processes are composed of multitiered signaling cascades of kinases starting from ligand binding to extracellular domains of RTKs that activate the entire pathways through tyrosine phosphorylation of the receptors and downstream effectors. A previous study reported that, based on proteomics data, RTKs were a major candidate target for osteosarcoma. In this study, activation profiles of six candidate RTKs, including c-Met, c-Kit, VEGFR2, HER2, FGFR1, and PDGFRα, were directly examined from chemonaive fresh frozen tissues of 32 osteosarcoma patients using a multiplex immunoassay. That examination revealed distinct patterns of tyrosine phosphorylation of RTKs in osteosarcoma cases. Unsupervised hierarchical clustering was calculated using Pearson uncentered correlation coefficient to classify RTKs into two groups—Group A (c-Met, c-Kit, VEGFR2, and HER2) and Group B (FGFR1 and PDGFRα)—based on tyrosine phosphorylation patterns. Nonactivation of all Group A RTKs was associated with shorter overall survival in stage IIB osteosarcoma patients. Percentages of tumor necrosis in patients with inactive Group A RTKs were significantly lower than those in patients with at least one active Group A RTK. Paired primary osteosarcoma cells with fresh osteosarcoma tissue were extracted and cultured for cytotoxicity testing. Primary cells with active Group A RTKs tended to be sensitive to doxorubicin and cisplatin. We also found that osteosarcoma cells with active Group A RTKs were more proliferative than cells with inactive Group A RTKs. These findings indicate that the activation pattern of Group A RTKs is a potential risk stratification and chemoresponse predictor and might be used to guide the optimum chemotherapy regimen for osteosarcoma patients.

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Introduction

Osteosarcoma is an aggressive primary bone sarcoma that occurs predominantly in children and teenagers [1]. Current treatment strategies for osteosarcoma include surgery to remove the tumor and chemotherapy [2]. The most effective regimen is based on a

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combination of methotrexate, cisplatin, and doxorubicin (MAP). Unfortunately, not all patients have a good response to the chemotherapeutic treatment; many patients with high-grade osteosarcoma develop chemoresistance to the MAP regimen, leading to poor clinical outcomes [3,4]. Additionally, conventional chemotherapy can cause various side effects that worsen patient outcomes. The ability to identify patients who will respond poorly to chemotherapy is a promising approach for treating patients more effectively and with less toxic effects.

In our previous work, we established a list of target proteins of FDA-approved drugs based on results reported in several proteomics studies of osteosarcoma [5]. Interestingly, it was found that receptor tyrosine kinases (RTKs) including fibroblast growth factor receptor 1 (FGFR1), platelet-derived growth factor receptor alpha (PDGFRα), mast/stem cell growth factor receptor Kit (c-Kit), vascular endothelial growth factor receptor 2 (VEGFR2), hepatocyte growth factor receptor (c-Met), and receptor tyrosine-protein kinase erbB-2 (HER2) were a major target group. Expression levels of these RTKs were higher in osteosarcoma cells compared to osteoblastic cells.

Since the discovery of the first RTK in 1978, RTKs have been shown to be important growth factor receptors that regulate critical cellular processes including cell survival, proliferation, differentiation, metabolism, cell-cell communication, cell migration, and cell-cycle control [6,7]. The human RTK family includes 58 members which fall into 20 subfamilies [8]. All known RTKs share a conserved molecular architecture with extracellular ligand-binding domains, a single transmembrane region, and a cytoplasmic kinase domain that is activated by tyrosine phosphorylation upon dimerization or oligomerization [8].

The FGFR family is comprised of four main members including FGFR1, FGFR2, FGFR3, and FGFR4 [9]. The extracellular region of all FGFRs contains three Ig-like domains that bind to FGFs in the presence of the accessory molecule heparin [10]. The main result of activation of FGF signals is to trigger the RAS–mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)–AKT signaling pathways which subsequently induce cell proliferation and cellular survival, respectively [11].

The PDGFR family includes PDGFR, Kit, CSF1R, and Flt3 which contain different numbers of extracellular Ig-like domains [10]. Binding of homodimeric or heterodimeric PDGFR (PDGFR-α an PDGFR-β) to different PDGFs (PDGFA, PDGFB, PDGFC, and PDGFD) transduces various signals and generates broad biological functions under diverse physiological and pathological conditions [12,13]. A system of the PDGFR/PDGF complex induces cancer cell proliferation, angiogenesis, metastasis, and the development of tumor-associated fibroblasts [12,14]. c-KIT receptor is also a member of the PDGFR family. That receptor binds to stem cell factor (SCF) molecules at the Ig-like domains of c-KIT [8]. Upon activation, the c-KIT/SCF system triggers various downstream pathways, mainly MAP kinase, that regulate cell survival and proliferation [15].

The VEGFR family (VEGFR1, VEGFR2, and VEGFR3) expresses Ig-like extracellular domains which are structurally related to the PDGFR family [16]. The VEGFR receptor and their co-receptors bind to distinct VEGF ligands including VEGFA, VEGFB, VEGFC, VEGFD, and placenta growth factor [17]. This different binding of the VEGFR/VEGFs regulates various biological mechanisms, mainly angiogenesis, vasculogenesis, lymphangiogenesis, permeability, inflammatory cell recruitment, and fatty acid uptake.

The c-MET receptor, also known as the hepatocyte growth factor receptor (HGF receptor), contains three extracellular domains including the Sema, the PSI, and the IPT domains [18]. Upon binding of the c-MET receptor and HGF, the complex triggers diverse signaling cascades, mainly the MAP kinase, the PI3K-Akt axis, the signal transducers and activators of transcription (STAT) pathway, and the IκBα–NF-κB complex [19–22]. The activation of c-MET induces tissue remodeling as well as promoting cell survival, proliferation, and migration that facilitate invasive growth and metastasis of cancer cells.

HER2 receptor (erbB2, HER2/neu) is a member of the human epidermal growth factor receptor family that also includes EGFR (HER1, erbB1), HER3 (erbB3), and HER4 (erbB4) [23]. The extracellular region of HER2 consists of four domains: domains I and III which comprise the β helix LRR-like "solenoid" domains as well as the cysteine-rich domains II and IV [8]. HER2 does not contain a ligand-binding domain, which makes this receptor highly active [24]. Activation of HER2 induces important biological mechanisms involved in cell survival, proliferation, and cell-cycle progression through the PI3K/AKT and RAF/MEK/MAPK pathways [24].

Phosphorylated RTKs subsequently recruit adaptor proteins to trigger various downstream cascade signaling. Much evidence indicates that aberrant activation of RTKs, including gene amplification, receptor overexpression, autocrine activation, and gain-of-function mutations, has been causally linked to cancer [25]. For example, in gastric, lung, and esophageal tumors, it was found that the cancers with *MET* gene amplification were more sensitive to MET kinase inhibitors than those not carrying this aberrancy [26,27]. Other examples include breast cancer with *ERBB2* gene amplification, GIST with *PDGFRα* gene mutation, GIST with *c-KIT* gene mutation, and CML with *FGFR1* gene translocation. The subset of patients with these aberrancies is highly sensitive to particular RTK inhibitors [28].

In this study, we examined further the activation state of those candidate RTKs directly in clinical samples (frozen osteosarcoma tissues) using the multiplex immunoassay, a rapid and clinically applicable method. The results of this experiment provide a better understanding of the relationship between activated RTKs and clinical outcomes in addition to identifying distinct groups among osteosarcoma patients. Moreover, the activated RTK patterns provide potentially important clues for further development of tailored therapy for the treatment of osteosarcoma.

Materials and Methods

Patients and Tissue Samples

A total of 32 patients diagnosed with stage IIB osteosarcoma and treated at Maharaj Nakorn Chiang Mai Hospital, Thailand, between 2010 and 2016 were included in this study. Patients were followed up for survival analysis for at least 24 months (until 30 June 2016). All biopsy samples were obtained prior to neoadjuvant treatments and were separated into three parts: one was kept as formalin-fixed, paraffin-embedded tissues for clinical diagnosis; the second was stored as fresh frozen tissues and used for determination of tyrosine phosphorylation of RTKs; and the third was processed further and cultured as primary cells. Tissue specimens were freshly frozen at −80°C within 30 minutes of surgery and stored until use.

To evaluate the percentage of tumor necrosis, tissue samples were obtained after the patients had been treated with chemotherapy. The