Identification of F-box/LLR-repeated Protein 17 as Potential Useful Biomarker for Breast Cancer Therapy

GARY GUISHAN XIAO[1,2,5], BING-SEN ZHOU[1], GEORGE SOMLO[3], JANA PORTNOW[3], AGNES JUHASZ[1], FRANK UN[1], HELEN CHEW[4], DAVID GANDARA[4] and YUN YEN[1]

[1]Department of Clinical & Molecular Pharmacology, City of Hope National Cancer Center, Duarte, CA;
[2]Department of Pediatrics/Mass Spectrometry Lab Facility,
Harbor-University of California Los Angeles, Torrance, CA;
[3]Department of Medical Oncology,
[4]University of California Davis Cancer Center, Sacramento, CA;
[5]Genomics & Functional Proteomics Laboratory, Osteoporosis Research Center,
Creighton University, Omaha, NE, U.S.A.

Abstract. *Background: The expression and activity of ribonucleotide reductase (RR) has been associated with resistance to multiple drugs in human cancer. The use of antisense oligonucleotide drug, GTI-2040, a 20-mer phosphorothioate oligonucleotide complemented to the human RR M2 subunit mRNA, represents an effective strategy for inhibiting RR. The increased specificity due to the anti-resistance effect of GTI-2040 may also lead to a more favorable therapeutic outcome. Materials and Methods: To understand the molecular mechanism underlying RR inhibition, patients' blood samples were analyzed using multiple dimensional proteomics technology via matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectrometry. Results: A major difference occurred at 5k m/z in the MALDI profile, which appeared only in the non-responsive group and diminished after GTI-2040 treatment. This specific peptide peak remained at the basal level in responsive patients. The peak was identified to represent the F-box/LLR-repeat protein 17 (FBXL17) through nanoelectrospray ionization liquid chromatography-tandem mass spectrometry (nanoESI LC-MS/MS). Further characterization revealed that FBXL17/SKP2 directly interacts with the human RR M2 (RRM2) subunit to promote hRRM2 overexpression in the breast cancer cell line MCF-7. Conclusion: Validation of this protein using real-time RT-PCR indicates the F-box protein 17 (FBXL17) can serve as a therapeutic target and surrogate marker for breast cancer therapy.*

Chemotherapy plays a major role in cancer treatment; yet, drug resistance hampers drug efficacy significantly. It is important to develop strategies to enhance drug efficacy. Antisense oligonucleotide drug therapy represents one of many novel target-specific cancer treatment strategies under development. Theoretically, antisense drugs can inactivate mRNA to inhibit protein expression through many potential mechanisms including steric hinderance of the mRNA interaction with ribosomes, spliceosomes or regulatory binding proteins, formation and degradation of RNA/DNA hybrids, and formation of DNA triplexes and prevention of transcription (1, 2).

Ribonucleotide reductase (RR) is an S-phase-specific, rate-limiting enzyme of the DNA synthesis pathway (3). RR plays a crucial role in providing a balanced supply of precursors for DNA synthesis and repair intracellularly (4). The human RR enzyme consists of two subunits, RRM1 and RRM2. Recently, the gene encoding a new RR family member, p53R2, was cloned, which contains a p53-binding site in intron 1 and encodes a 351 amino acid polypeptide with striking similarity to hRRM2 (5).

The expression and activity of RR is known to play a pivotal role in resistance to many drugs in human tumor cells (3). Yet, many currently used cytotoxic agents that inhibit RR lack specificity. The antisense oligonucleotide, originally designed to hybridize to RRM2 mRNA specifically, is

expected to be more effective in inhibiting the target and its increased specificity may lead to a more favorable therapeutic outcome. Studies on the role of RRM2 protein in determining the malignant potential of cells suggest that the use of a specific inhibitor of the RRM2 mRNA may exert antineoplastic effects *via* multiple signaling pathways (6).

Pre-clinical data from colony-forming assays have shown the ability of this antisense drug to inhibit the growth of several human tumor cells (5). Currently, clinical trials of antisense drug are being conducted by the NCI in the United States and Canada. Based on the trend in antisense therapeutic development towards combination therapy with standard chemotherapeutic drugs and the dramatic anti-tumor activity of GTI-2040 in combination with a number of agents in pre-clinical models of human cancer (7), these trials were designed for combination treatments. These studies incorporate correlative science components to assess whether target down-regulation correlates with clinical outcome and whether the effect on RRM2 levels in WBCs correlates to RRM2 levels in tumor samples. A series of combinational therapies of GTI-2040, with capecitabine in metastatic breast cancer (current study), with oxaliplatin and capecitabine in metastatic colorectal cancer, with single agent gemcitabine in solid tumors, with docetaxel in advanced non-small cell lung cancer and hormone refractory prostate cancer, with cytarabine in acute myeloid leukemia, were performed. However, diversified clinical outcomes have been observed, only small portion of participated patients had a response to the combo therapy (data not shown). To resolve the discrepancy above, we selected patients' plasma samples from one of the trials (metastatic breast cancer) mentioned above in order to get more insight into the mechanism underlying the target therapies.

Multiple dimensional proteomics technology (MudiPT) is a powerful tool that can directly analyze whole proteome expression as well as modification at the post-translational level (8-10). Since transcriptional regulation is often difficult to relate to protein abundance, proteomics has gained interest among investigators as it permits the qualitative and quantitative assessment of a broad spectrum of proteins in human plasma that can be related to various diseases (11-12). In this study, to help us better understand the molecular mechanism underlying RR inhibition and the corresponding clinical outcome, patients' blood samples were collected at the City of Hope Comprehensive Cancer Center and were analyzed using multiple dimensional proteomics technology *via* matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectrometry. As compared to the sensitive group, a major difference at 5k m/z in the MALDI profile was observed in the plasma samples from the less sensitive group. The peak was identified to represent the F-box/LLR-repeat protein 17 (FBXL17) that may serve as a therapeutic target and surrogate marker for breast cancer therapy.

Materials and Methods

Patient sample. All patients gave signed consent for the IRB-approved treatment protocol. 15 patients were participated in this study based on inclusion criterion as follows: (a) Patients must have histologically or cytologically confirmed metastatic adenocarcinoma of the breast. (b) Patients must have measurable disease. (c) Patients must have progressed on at least one but no more than two prior chemotherapy regimens for metastatic disease. (d) Age >18 years; Metastatic adenocarcinoma of the breast is not expected to occur in patients less than 18 years. (e) Life expectancy of greater than 3 months; (f) ECOG performance status <2. (g) Patients must have normal organ and marrow function as defined as: leukocytes >3,000/μL, absolute neutrophil count >1,500/μL, platelets >100,000/μL, total bilirubin must be within normal institutional limits, AST(SGOT)/ALT(SGPT) <2.5 X institutional upper limit of normal, creatinine within normal institutional limits or creatinine clearance >60 mL/min. (h) Patients must have completed radiation treatment > 4 weeks prior to study entry. Previously radiated area(s) must not be the only site of disease. (i) All major surgical procedures must be completed >4 weeks prior to study entry. Placement of vascular access device or tissue biopsy will not be considered major surgery. (j) Women of child-bearing potential and men must agree to use adequate contraception (hormonal or barrier method of birth control; abstinence) prior to study entry and for the duration of study participation. Should a woman become pregnant or suspect she is pregnant while participating in this study, she should inform her treating physician immediately. (k) Ability to understand and the willingness to sign a written informed consent document. (l) Patients must agree to the placement of a central venous catheter in order to receive the continuous infusion treatment. The blood samples were collected from all patients before treatment (day 1) and after treatment for 1 day, 6 days, and 14 days, according to the protocol using sodium citrate CPT tubes. Blood samples used in this proteomics study were only selected from two time points for each patient blood sample, which are before treatment (day 1) and after treatment (day 2) for comparison. To achieve >95% statistical power (13), 12 samples were selected for mass spectromic analysis. Blood samples were centrifuged at 2,500 rpm at 4°C for 5 min, sera were then collected and saved at –80°C until use.

Plasma protein preparation. High abundant proteins in plasma sample were depleted in 20% acetonitrile (ACN) combined with 2% trichloride acid (TCA) at –20°C for one hour. The protein solution then was clarified and the supernatant was divided into two fractions: 1/3 fraction was passed through a C_{18} zip tip (Millipore, Bellerica, USA) and eluted with 50% ACN and 0.1% TFA before use of MALDI profiling. The remaining 2/3 fraction was desalted, lyophilized, and used for nanoLC MS/MS analysis.

Plasma protein identification by mass spectrometry. For matrix-assisted laser desorption and ionization (MALDI) profiling, depleted plasma samples were spotted onto MALDI targets. MALDI peptide fingerprint mass spectra were acquired with a MALDI time-of-flight instrument (MALDI-DE STR workstation; Applied Biosystems, Foster City, USA), using α-cyano-4-hydroxycinnamic acid (Sigma, St. Louis, USA) as the matrix. For peptide sequencing, the depleted plasma samples were first separated on 10-20% Tricine gel (1-D gel) (Invitrogen, Carlsbad, USA). The bands of interest were excised, destained, washed and extracted from the gel using the protocol described elsewhere (8). Measurements of the extracted

peptide mixtures from 1-D-gel bands were performed on a ThermoFinnigan nanoESI LCQ mass spectrometer (San Jose, USA), equipped with a nanoflow electrospray ionization (nanoESI) interface operated in the positive ion mode at the Core Facility of LA Biomedical Institute-Harbor UCLA medical center (Carson, Torrance, USA). The instrument was controlled using TunePlus 2.0 (beta 3) (San Jose, USA), and the acquired spectra were evaluated using Xcalibur 2.0 software (San Jose, USA). Acquired MS/MS spectra from LCQ experiments were sought with SEQUEST against the National Center for Biotechnology Information (NCBI) protein database using the SEQUEST search program (14). Positive protein identification was based on standard SEQUEST criteria (Q score) for statistical analysis of the LC-MS/MS data (14).

SKP2 construction. Human SKP2 cDNA was generated by reverse transcription polymerase chain reaction (RT-PCR) from HepG2 cell line total RNA using the following primers: 5'-AATCTGGG AGGCGAGCAGCT-3' and 5'-CAATTGGGCTTCCT GCCTATTT-3'. The open reading frame (ORF) sequence of SKP2 was inserted in frame into pCMV-Tag1 vector with the following linkers using the above RT-PCR product as template: 5'-TGGCGGCCGCCACCA TGCACAGGAAGCACCTCCA-3' and 5'-ATAAGCTTTAGACAA CTGGGCTTTTGCAG-3'(*Not*I and *Hind*III sites incorporated into sense and antisense primers, respectively, are underlined). The PCR product and vector were digested with *Not*I and *Hind*III separately, and ligated together. SKP2 coding sequence was also inserted into pBind and pACT vectors with the same strategy, except the *Eco*RV and *Not*I sites were incorporated into the sense and antisense primers. Full-length human RRM2 cDNA sequences were amplified by PCR using PCR Master Mix (Cat# M7502; Promega, San Luis Obispo, USA). Forward and Reverse Primer sequences used in PCR were 5'-TCGCCAGGGATCCATAT GCTCTC-3' and 5'-ACCTGCGGCCGC TTAGAAGTCAG-3', respectively. All resulting PCR products were BamHI/NotI double-digested and inserted into BamHI/NotI-opened pACT-VP16 or pBind-Gal4 plasmids (Cat# E2440; Promega), resulting in expression constructs pACT-VP16/RRM2 and pBind-Gal4/RRM2. All constructs were verified by sequencing. All PCRs were carried out with proofreading *Pfu* DNA polymerase. All constructs were further verified by DNA sequencing.

Cell culture and SKP2 transfection in MCF-7. MCF-7 human breast cancer cells were purchased from the American Type Culture Collection (ATCC; Manassas, USA). The cells were seeded to achieve 85% confluence on the day of transfection. Transient transfection of SKP2 was performed using Oligofectamine 2000 following the protocol recommended by the manufacturer (Invitrogen, Carlsbad, USA). All the transfectants were maintained in 10% fetal bovine serum (FBS)/Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Inc., Herndon, USA) until collected for analysis.

Immunoprecipitation and Western blot. Immunoprecipitation assays were described in our previous paper (15). Each immunoprecipitate (30 μl) was separated on 4-20% SDS-PAGE and transferred onto a PVDF membrane (GE Healthcare Bio-Sciences Corp, Piscataway, USA). Immunoblotting was performed by incubation with the corresponding antibodies using the procedure as follows. The blots were blocked in 1% I-block™ blocking buffer (Applied Biosystems). Following sequential blockage with primary (1:200 diluted) (goat anti-RRR2, goat anti-SKP2, and goat anti-p53R2) and secondary (1:2000 diluted) (anti-goat IgG) antibodies (Santa Cruz

Biotechnologies, Santa Cruz, USA), a thin layer of CSPDR ready-to-use substrate solution (Applied Biosystems) was applied over the membrane. After incubation for 5 min, the membrane was exposed to an X-ray film.

Fluorescence microscopy. Cells were grown on sterile glass coverslips at 37°C for 24 h, transfected with SKP2 plasmid and continuously incubated for another 48 h before fixing with methanol at –20°C for 20 min. The rehytrated cells were incubated with 2 μg/ml primary antibodies (Santa Cruz Biotechnologies) of SKP2 (goat) and RRR2 subunit (rabbit) for 1 h. Cells were then washed briefly with phosphate-buffered saline (PBS) three times before incubating with either FITC-conjugated anti-goat (5 μg/ml) or rhodamine-conjugated anti-rabbit (5 μg/ml) secondary antibody (Santa Cruz Biotechnologies) in a dark chamber for 45 min. Cells were then washed 3 times with PBS and restained with 4'-6-diamidino-2-phenylindole (DAPI) for 1 min. Glass slides were then mounted with a coverslip with 90% glycerol in PBS and then observed under the confocal microscope (Carl Zeiss LSM 510 NLO; Thornwood, USA) with a Plan-Apochromat 100×1.4 DIC oil objective.

RR Activity assay. The RR activity assay was performed as described elsewhere (15). In brief, MCF-7 cells (1×10^7) were placed in a 100 mm dish and incubated for 24 h. Cells were then transfected using the protocols mentioned above. Cells were washed and detached from the plate, then pelleted at 300 ×g for 10 min at 4°C. The pellets were washed again with PBS. One volume of low salt homogenization buffer (10 mM HEPES pH7.2, 2 mM DTT) was added to the cell pellets and the cell suspension was passed through a needle 20 times on ice. After homogenization, one volume of high salt buffer (1 M HEPES, pH 7.2, 2 mM DTT) was added and the cell suspension was again passed through a needle 20 times on ice. Cell debris was removed by centrifugation at 16,000 ×g at 4°C for 20 min. The supernatant was passed through a Sephadex G25 spin column (GE Healthcare Bio-Sciences Corp, Piscataway, USA) pre-equilibrated with buffer (50 mM of HEPES, pH 7.2, with 2 mM of DTT) to remove endogenous nucleotides. The protein concentration was measured by the Bio-Rad protein assay (Bio-Rad, Hercules, USA). The reaction mixture contained 0.15 mM [^{3}H] radioactive cytidine diphosphate (rCDP) (0.02 μCi), 2 mM adenosine triphosphate (ATP), 0.05 mM CDP, 50 mM HEPES (pH 7.2), 6 mM dithiothreitol (DTT), 4 mM magnesium acetate, and differing amounts of cell extract in a final volume of 0.15 mL. After a 20 minute incubation, dCDP formed was dephosphorylated by phosphodiesterase and C and dC were separated by HPLC with a C_{18} ion exchange column. The C and dC peaks were collected in scintillation vials and the amount of radioactivity was measured with a Beckman LS 5000CE liquid scintillation counter (Beckman Instruments, Berkeley, USA). Specific activity was calculated as nmol CDP/h/mg protein.

Mammalian two hybridization (MTH). Mammalian two hybridization screening was carried out according to the manufacturer's protocol for CheckMate™ mammalian two hybridization system (Promega). Dual-Luciferase® Reporter Assay System (Promega) was used to measure the extent of interaction.

RNA isolation and cDNA synthesis. RNA isolation and cDNA synthesis were performed using an established protocol (16). Human peripheral white blood cells (WBC) were prepared from fresh blood samples drawn before and after administration of the

Table I. *Real-time PCR primers and probes*.

Gene	Sequence	GenBank number
β-Actin	Forward: 5'-CGAGCGCGGCTACAGCTT-3'	NM_00101 (655-713bp)
	Reverse: 5'-CCTTAATGTCACGCACGATT-3'	
	Probe: 5'TET-ACCACCACGGCCGAGCGG-3'TAMRA	
Skp2	Forward: 5'-AGTTGCAGAATCTAAGCCTGGAA-3'	NM_032637 (796-869)
	Reverse: 5'-ATTTGAGTTTTTTGCGAGAGTATTGA-3'	
	Probe: 5'FAM-CCTGCGGCTTTCGGATCCCATT-3'TAMRA	
18S	Forward: 5'-AACGAGACTCTGGCATGCTAACTA-3'	M10098 (1483-1562bp)
	Reverse: 5'-CGCCACTTGTCCCTCTAAGAA-3'	
	Probe: 5'TET-TACGCGACCCCCGAGCGGT-3'TAMRA	

drug. Erythrocytes were removed with Erythrocyte Lysis Buffer (Qiagen, Inc. Valencia, CA, USA) according to the manufacturer's instructions, and WBC pellets were snap frozen and kept at –80°C until processed further. The total RNA was isolated using RNeasy® Micro Kit (Qiagen Inc.) according to the manufacturer instructions. Genomic DNA contamination was removed with DNAse I treatment on the column. The integrity of isolated RNA was tested on 1% agarose gel (SeaKem, FMC, Rockland, ME, USA) or with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The RNA concentration and A_{260}/A_{280} ratio was measured with a UV spectrophotometer. cDNA was prepared from total RNA in a 25 μl volume, using MMLV reverse transcriptase enzyme and random hexamers as primers (Invitrogen) as described elsewhere (16). Quantification of gene expression was carried out using cDNA samples and a real-time PCR method. A set of primers and probes for each of RRM1, RRM2, TBP and RPL2 were purchased from Applied Biosystems, Foster City, CA, USA (pre-designed Gene Expression Assays). Additional primers and probes for 18S and α-actin were designed according to the Applied Biosystems guidelines (Primer Express software; Applied Biosystems) to fit the real-time PCR requirements as described elsewhere (16). Genomic DNA amplification was excluded by designing primers around the exon-intron splicing sites. The sequences of primers and probes are shown in Table I.

Assessment of F-box protein 17 (Skp2) mRNA levels by TAQMAN RT-PCR. Real-time PCR was performed using an ABI 7900HT Sequence Detection System (Applied Biosystems) using an established protocol (17). For target genes using in-lab designed primer/probe sets, the probe concentration was 0.3 μmol/l and primer concentrations for the α-actin gene and 0.2 μmol/l for the 18S gene. For assays containing the ABI Gene Expression Assay primer/probe sets, 1 μl primer and probe mix (20 × concentration) was added to the reaction. The PCR amplification was performed on a 384-well plate using the default cycling conditions. During the PCR reaction, the Taq DNA polymerase cleaved the probe and released the 5'end reporter fluorescence dye [6-carboxy-fluoresine (FAM), tetrachloro-6-carboxy-fluoresine (TET)] whose fluorescence was detected by the ABI 7900HT Sequence Detection System (Applied Biosystems). cDNA sample from normal leukocyte total RNA (BD Biosciences, Palo Alto, CA, USA) was used as positive control. Relative gene expression was determined as the ratio of the gene of interest to the internal reference gene expression based on the Ct values (17).

Results

Identification of F-box/LLR-repeated protein 2 (Skp2) from plasma of breast cancer patents. To determine why certain breast cancer patients are less sensitive to GTI-2040 treatment, we employed multiple fractionational step to detect the difference in proteome of patients' serum samples before versus after the treatment. To eliminate the interference of high abundant proteins in sera with the proteomics analysis, 2% trichloric acid (TCA)/20% acetonitrile (ACN) was used to precipitate sera, which were collected from 12 breast cancer patients. We found that ~80% of major abundant serum proteins were removed (data not shown). The depleted serum sample was further purified with C_{18} zip tip and then spotted onto MALDI plate for direct proteome profiling with a scan range of 500 to 7,500 m/z. The results from 12 serum samples in the less sensitive group showed that at least one peak at 5033.70 m/z was lost after GTI-2040 treatment (Figure 1A, upper panel) when compared to before the treatment (Figure 1A, below panel), whereas this peak was not detected in the sensitive group.

To verify these results, the depleted plasma samples from the less sensitive group were also analyzed by using 10-20% Tricine/glycine gel (Invitrogen) electrophoresis. The results showed in Figure 1B. We observed a 5 kDa-band in the gel, which is similar size to that in mass spectrum (Figure 1B). We estimated the abundance of the protein band of 5 kDa that is less than 0.1% over SYPRO Ruby-staining intensity (data not shown).

To further identify this protein, we excised the 5 kDa-band from the gel and performed in-gel digestion using the established protocol (9). Using a scoring procedure for measuring the quality of the results for protein identification obtained from spectral matching of MS/MS data using the SEQUEST database search program (14), we identified this fraction as F-box/LLR-repeated protein 17 (the variants of S-phase kinase protein 2) (FBLX 17/Skp2) (Figure 2).
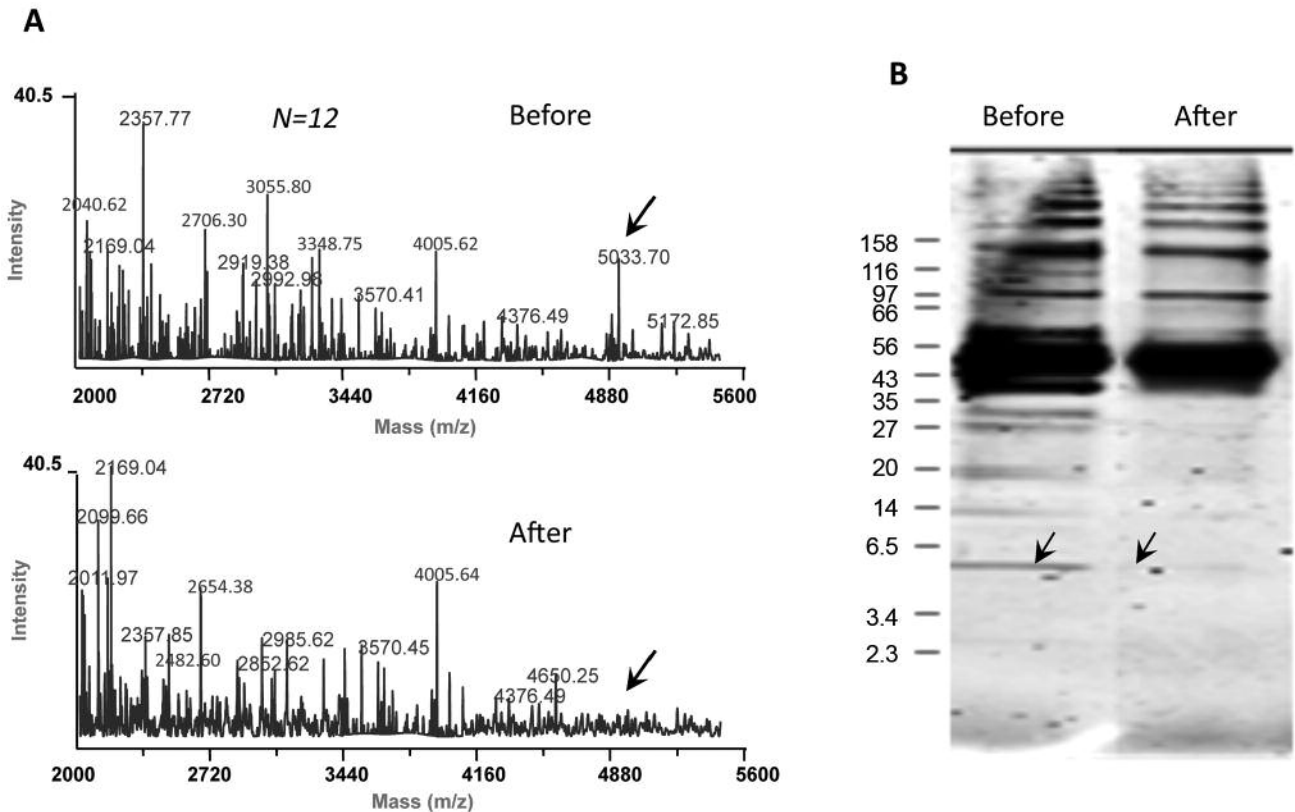
Figure 1. *Detection of F-box/LLR-repeated protein 2 (Skp2) from breast cancer patients of non-sensitive group before and after treatment. A, MALDI-TOF profile of patients' plasma. Patient plasma sample was depleted before MS profiling. MALDI profile was performed in Voyager-DE STR workstation with mass range of 1,000-10,000 m/z. Up to 12 patients from non-sensitive group of breast cancer were analyzed. B, Plasma protein profiling in SDS-Tricine gel. 150 μg of depleted plasma protein from the less sensitive group before and after GTI-2040 treatment was separated on 10-20% Tricine/glycine, then stained with fluorescent dye SYPRO Ruby. The gel image was acquired by FX UV scanner (Bio-Rad, CA, USA). The experiments were performed in triplicate. Details are shown in the Materials and Methods.*

Synchronously expression of F-box/LLR-repeated protein 2 (Skp2) and hMMR2. To determine the relationship between F-box/LLR-repeated protein 2 (Skp2) and hMMR2, we first examined the expression pattern of SKP2 and RRM2 in the hydroxyurea-resistant cell line KBHURs and found that both were synchronously over-expressed (Figure 3A). This association was also confirmed in a KB cell line stably transfected with RRM2 gene (Figure 3A).

To further confirm the association between SKP2 and RRM2 in breast cancer cells, we subcloned the full-length SKP2 gene into pcMyc-Tag vector and transfected into the breast cancer cell line MCF-7 to examine whether SKP2 can regulate RRM2, resulting in an altered RR activity. The results from immunoblotting assays showed that SKP2 over-expression enhances the production of RRM2 (Figure 3B). We also monitored the expression of p53R2 (as it shares striking similarity to RRM2) yet observed no such effect on p53R2 (Figure 3B).

To understand the nature of interaction between SKP2 and RRM2, we examined the distribution pattern of SKP2 and RRM2 in breast cancer cells. MCF-7 cells were transiently transfected with the SKP2 gene, and subjected to indirect immunofluorescence assay utilizing goat anti-SKP2 and rabbit anti-RRM2 antibodies as the primary antibodies, followed by FITC-conjugated anti-goat IgG and rhodamine-conjugated anti-rabbit IgG as the secondary antibodies (Figure 3C). In SKP2 transfected cells, SKP2 (green) and the over-expressed RRM2 (red) were co-localized in the cytosol (Figure 3C).

Interaction of F-box/LLR-repeated protein 2 (Skp2) and RRM2 subunit regulates activity of RRM2. To determine whether the SKP2's association with RRM2 occurs through a direct interaction between SKP2 and RRM2, two distinct approaches were employed. Firstly, we used either SKP2 or RRM2 as bait to "fish" RRM2 or SKP2 in SKP2 gene-transfected breast cancer cells; then the protein complex formed by fishing was separated by SDS-PAGE and electrotransferred onto a PVDF membrane. Immunoblotting assay was performed to detect RRM2 or SKP2 on the membrane. We observed a strong signal of RRM2 or Skp2
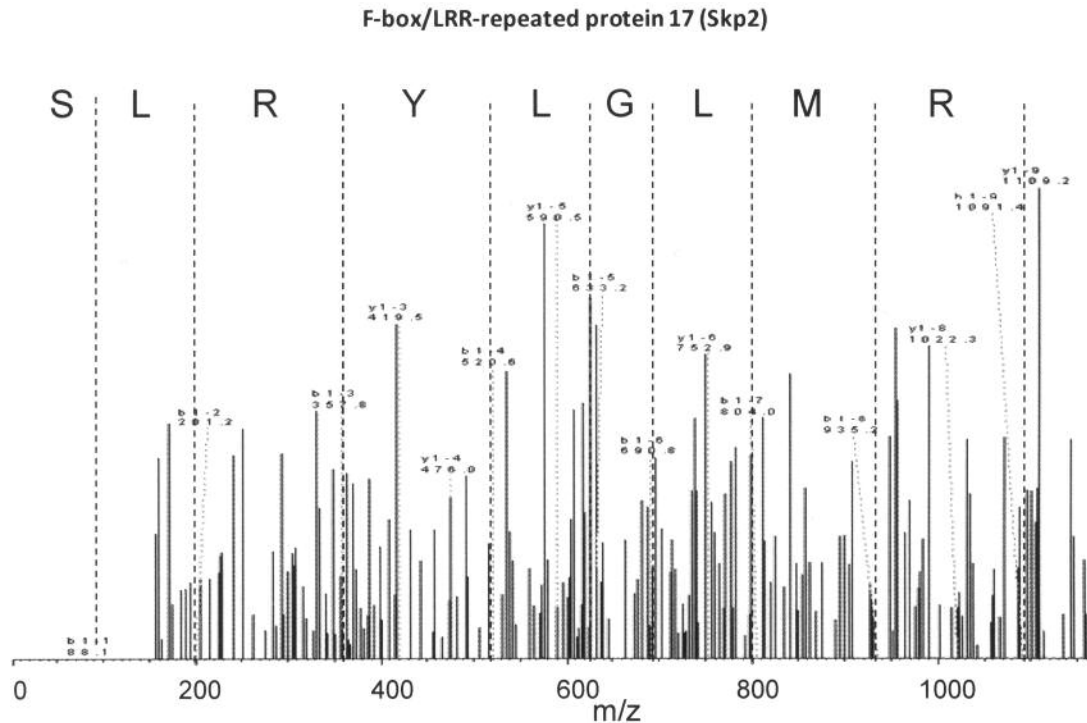
Figure 2. *Identification of FBXL17/Skp2 by mass spectrometry. A 5 kDa protein band on the gel was stained using Sypro Ruby, excised, digested by trypsin, and further identified using nano-ESI LC quardruple ion trap MS/MS (LCQ). Using a SEQUEST data search, this 5 kDa protein band was identified as SKP2 (SLRYLGLMR). To ensure reproducibility, all experiments were repeated 3 times. Detailed information is provided in Materials and Methods.*

if used either Skp2 or RRM2 as a bait in Skp2-transfected MCF-7 cells. These results suggested that SKP2 directly interacts with RRM2 in MCF-7 cells (Figure 4A).

Secondly, we utilized CheckMate™ mammalian two-hybridization system (Promega) (18) to confirm the above results. SKP2 and RRM2 were subcloned into either pACT vector, which contains TAD, or pBIND vector, which contains DBD. If there is interaction between the two proteins, it will transactivate the reporter gene luciferase, allowing one to quantify the interaction. As controls, MCF-7 cells were co-transfected with either pBIND vector/pACT-SKP2/GAL 5-luciferase or pBIND-RRM2/pACT vector/GAL5 luciferase combination. Co-transfecting MCF-7 cells with pBIND-RRM2/pACT-SKP2/GAL5-Luciferase resulted in a significantly higher luciferase activity ($p<0.05$) (Figure 4B), indicating that SKP2 interacts with RRM2 directly. Next, we examined whether SKP2's interaction with RRM2 activates the RR activity. MCF-7 cells, which express a moderate level of RRM2 (Figure 3B), were transfected with SKP2 gene and the resulting RR activity measured. The results showed that the RR activity in SKP2-transfected cells is at least 1.5-fold higher than that in control cells (Figure 4C), indicating that the interaction of SKP2 with RRM2 enhances the RR activity. That the expression level of p53R2,

another subfamily member, is very limited in this cell line (Figure 3B) further supports this view.

Now that SKP2 can directly regulate hRRM2 subunit to enhance RR activity, we expect the RRM2 activity will be dramatically suppressed as the induction of GTI-2040. To test this hypothesis, we treated SKP2-transfected MCF-7 cell line with or without GTI-2040 at final concentration of 0.2 μM (Figure 5A). Untransfected cells without GTI-2040 treatment served as control. Due to interaction of SKP2 and RRM2, the RRM2 was enhanced in SKP2 transfected cells compared to control while decreased to basal level upon the induction of GTI-2040 (Figure 5A). On the other hand, SKP2 level in transfected cells also decreased significantly as the induction of GTI2040 (Figure 5A). These results suggested that GTI-2040 can interrupt the interaction between SKP2 and RRM2 by dissociation of SKP2-RRM2 complex.

To further examine the relationship between SKP2 and RRM2 in breast cancer patients, we extracted RNA from human peripheral white blood cells from fresh blood samples drawn before and after the administration of GTI-2040. We then determined the mRNA level of both SKP2 and RRM2 by analyzing 12 patients' RNA samples from both less sensitive and sensitive groups pre- and post-treatment using real time PCR. The results showed that the mRNA level of
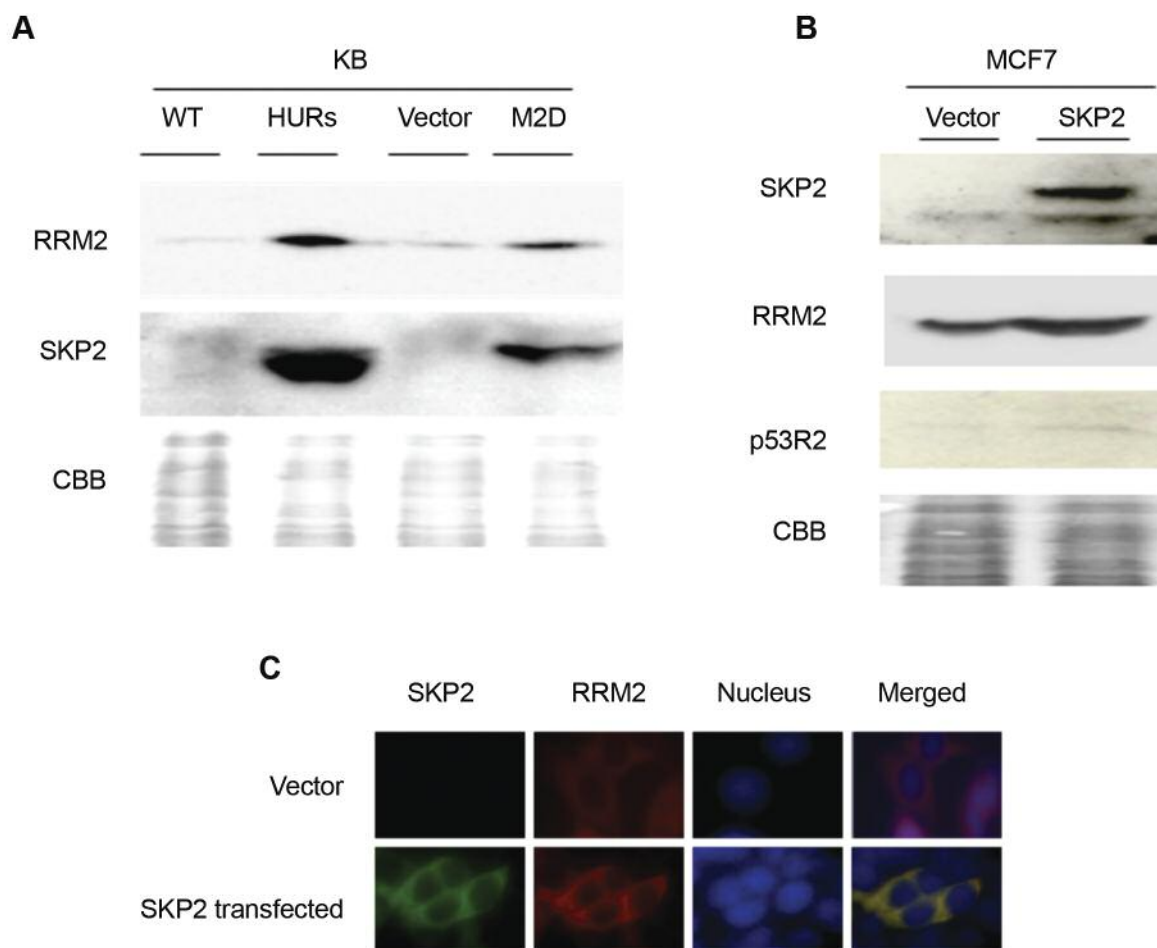
Figure 3. *Synchronized expression of SKP2 and RRM2 in both KB and MCF-7 cell lines. A, Western blot analyses of SKP2 and RRM2 were performed in both hydroxyurea-resistant (HURs) and RRM2 stable transfectant KB cell lines. B, Western blotting analyses of SKP2, RRM2, and p53R2 were performed in the breast cancer cell line MCF-7. C, Fluorescence microscopy demonstrated that both SKP2 (labeled with FITC) and RRM2 (labeled with rhodamine) were co-localized in the cytosol of MCF-7 cells. Experiments were performed in triplicates. Details are given in Materials and Methods.*

RRM2 remains unchanged in less sensitive group – as with SKP2 (data not shown). The level of RRM2 mRNA, however, decreased dramatically (>99%) upon drug treatment in sensitive group – as with the SKP2 mRNA (Figure 5B). Taken together, the results indicate that the mRNA level of SKP2 correlates with RRM2 in breast cancer patients.

Discussion

Plasma is a complicated system that regulates various biological processes (11). It has been reported that over 10,000 different proteins have been estimated to be commonly present in the plasma, most of which are at very low relative abundances (19). Their concentrations range more than 15 orders of magnitude. The dynamics of proteins in the plasma reflects physiological or pathological states of the human body. Therefore, it is generating great asset for scientists to make their efforts to discover biomarkers used for diagnostic or prognostic detection of many common malignant disease. Serum biomarkers can be distinguished into three different types: 1) serum proteins that are differentially expressed in patients with cancer, or serum proteins that are cleaved or modified in cancer patients, 2) proteins that are secreted by tumor cells into the circulation, 3) intra-cellular tumor proteins that are released when tumor cells die. As the development of proteomics, more and more proteomics techniques are employed to profile proteins existing in plasma and identify disease-related plasma markers (20). In the last 5 years, important progress has been made in characterization of the plasma proteome. This includes use of multidimensional peptide separation coupled
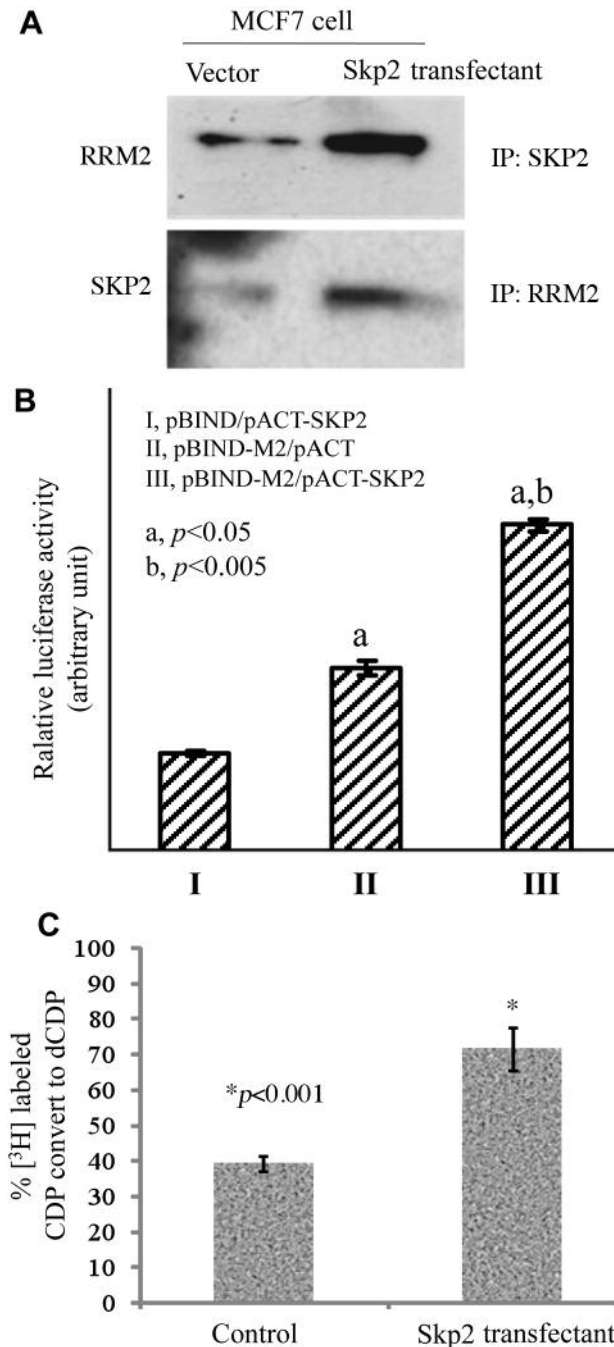
A

MCF7 cell

Vector   Skp2 transfectant

RRM2   IP: SKP2

SKP2   IP: RRM2

B

I, pBIND/pACT-SKP2
II, pBIND-M2/pACT
III, pBIND-M2/pACT-SKP2

a, $p < 0.05$
b, $p < 0.005$

Ralative luciferase activity (arbitrary unit)

a,b

a

I   II   III

C

% [^{3}H] labeled CDP convert to dCDP

*$p < 0.001$

*

Control   Skp2 transfectant

Figure 4. *Immunoprecipitation (IP) and mammalian two hybridization (MTH) demonstrated the interaction of SKP2 and RRM2 subunit in the MCF-7 cell line. A, Use of IP to demonstrate the interaction between SKP2 and RRM2 subunit in the MCF-7 cell line. MCF-7 cells were transfected with SKP2 construct, then either SKP2 or RRM2 were used as a 'bait' to pull-down the interacted proteins which were detected by Western blotting assays using SKP2 or RRM2 as probe. B, Mammalian two hybridization assay further confirmed the interaction between SKP2 and RRM2 subunit in MCF-7 cell line. Statistic analysis, II vs. I shows significance (a, p>0.05), III vs. I shows remarkable significance (b, p>0.005), III vs. II shows significance (a, p>0.05). C, RR activity was evaluated using [^{3}H] HPLC in the SKP2 transfected MCF-7 cell line. Experiments were performed in triplicates. Details are given in Materials and Methods.*

A

MCF7

Control   −GTI2040   +GTI2040

RRM2

SKP2

CBB

B

mRNA expression: Log$_2$ (relative mRNA abundance)

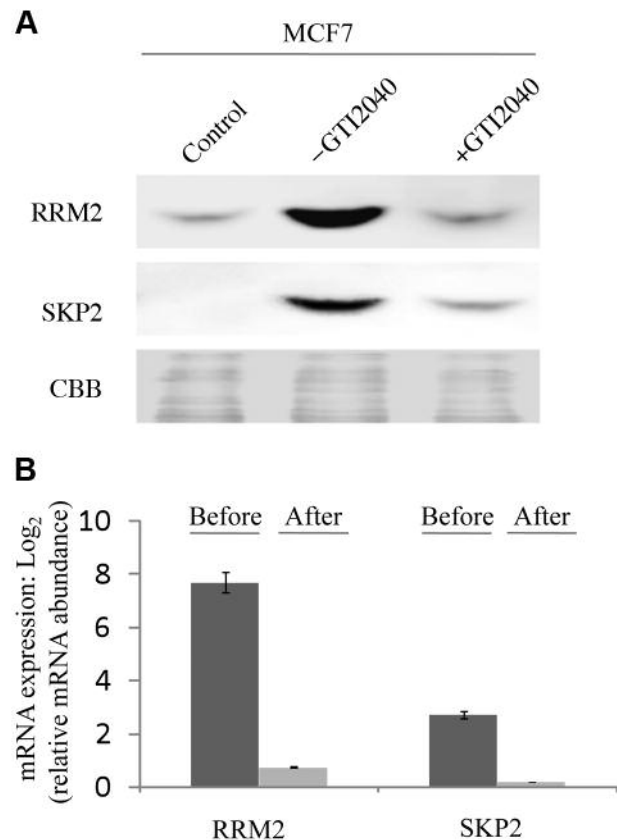Before   After   Before   After

RRM2   SKP2

Figure 5. *The inhibitory effects of GTI-2040 on RRM2 protein in the MCF-7 cell line and on RRM2 mRNA in breast cancer patients in the absence of SKP2. A. In vitro lipofectin-mediated SKP2 transfection of the human breast cancer cell line MCF-7 with 0.2 μM GTI-2040. SKP2-transfected cells were treated with GTI-2040 for 4 h before harvesting for protein expression assay. Steady-state levels of RRM2 protein were determined by Western blotting after GTI-2040 treatment. Untransfected cells serve as control that treated with lipofectin and transfection vector. The GTI-2040 effects were compared by treating SKP2 transfected cells with or without drug. B, SKP2 and RRM2 mRNA expression levels, in patient WBC samples before (the dark grey bar) and after (the light grey bar) GTI-2040 treatment, were measured by real-time RT-PCR. The results were normalized to actin as a 'housekeeping' gene. The arrow bars represent the SEM values from nine different individuals with triplicate measurements. Experiments were performed in triplicates. Details are given in Materials and Methods.*

with MS/MS and the creation of a human plasma protein database to lead to identifying new marker candidates for lung (21), ovarian (22), and prostate cancers (23). Despite recent excitement in the area of serum proteomics, any marker or marker concept always shows a lack of sensitivity due to the simple fact that some tumors are too small to release detectable amounts of protein. So far, there are still a number of proteins, especially low abundant species, which remain unknown in serum/plasma. The key issues are now to increase the sensitivity of detection for low abundance

proteins in plasma and perform an effective validation strategy on identified protein markers in order to minimize false positive error (24). Plasma proteins usually consist of a dynamic range of proteins in terms of their abundance, with small molecules with lower abundance generally executing important functions in specific biological processes. Since high abundance proteins (*e.g*., albumin, IgG) may interfere with our analysis of low abundance protein species, it was necessary to deplete them from the fractionated plasma samples using physical methods (*e.g.* filtration, precipitation). Efficient removal of high abundant proteins in plasma help to identify the F-box/LLR-repeat protein 17 (FBXL17, SKP2) in plasma samples from patients who have less responsive to GTI2040 treatment. Although there may be more differentiated peptides either suppressed or induced upon the drug treatment compared to before treatment, a fragment with 5 kDa was identically shown in both MS spectra and/or gel.

Identification of FBXL17/Skp2 has speculated a plausible interpretation for those who have different responsiveness to the antisense drug treatment. This may be interpreted by the following: 1) FBXL17/Skp2 can restore RRM2 subunit in both expression and activity through phosphorylation in cytosol, causing a high level of RRM2 in cytosol; 2) high level of Skp2/RRM2 complex in the cytosol could prevent RRM2 from translocating into the nucleus as cancer cells respond to GTI-2040 treatment, which causes the treatment to be in vain. For sensitive patients to the drug, RRM2 without Skp2 protection remains at a high level in the nucleus to produce more dNTPs for cancer cell proliferation. GTI-2040 can stop the dNTP production machinery by killing RRM2 activity in the nucleus as patients respond to drug treatment, which causes this group of patients to be highly sensitive.

What is the clinical implementation of FBXL17/Skp2? Analysis of mRNA levels in both sensitive and less sensitive groups as compared pre- and post-treatment suggests that the ratio of SKP2 mRNA to RRM2 mRNA level may be a useful index for drug resistance. Prior to GTI-2040 treatment, this ratio is close to 1.0 in less sensitive group whereas the ratio decreases to 0.25 in sensitive group (Figure 5B).

In clinical practice, identification of Skp2, which may be used as a target and surrogate therapeutic marker for breast cancer, could help clinicians to make good choice in treating breast cancer patients.

Acknowledgements

References

1 Buolamwini JK: Novel anticancer drug discovery. Curr Opin Chem Biol *3*: 500-509, 1999.

2 Gewirtz AM: Oligonucleotide therapeutics: a step forward J Clin Oncol *18*: 1809-1811, 2000.

3 Goan YG, Zhou B, Hu E, Mi S and Yen Y: Overexpression of ribonucleotide reductase as a mechanism of resistance to 2,2-difluorodeoxycytidine in the human KB cancer cell line. Cancer Res *59*: 4204-4207, 1999.

4 Xue L, Zhou B, Liu X, Qiu W, Jin Z, Yen Y: Wild-type p53 regulates human ribonucleotide reductase by protein-protein interaction with p53R2 as well as hRRM2 subunits. Cancer Res *63*: 980-986, 2003.

5 Desai AA, Schilsky RL, Young A, Janisch L, Stadler WM, Vogelzang NJ, Cadden S, Wright JA and Ratain MJ: A phase I study of antisense oligonucleotide GTI-2040 given by continuous intravenous infusion in patients with advanced solid tumors. Annals of Oncology *16*: 958-965, 2005.

6 Fan H, Villegas C, Huang A and Wright JA: Suppression of malignancy by the 3' untranslated regions of ribonucleotide reductase R1 and R2 messenger RNAs. Cancer Res *56*: 4366-4369, 1996.

7 Lee Y, Vassilakos A, Feng N, Lam V, Xie H, Wang M, Jin H, Xiong K, Liu C, Wright J and Young A: GTI-2040, an antisense agent targeting the small subunit component (R2) of human ribonucleotide reductase reductase, shows potent antitumor activity against a variety of tumors. Cancer Res *63*: 2802-2811, 2003.

8 Xiao GG, Nel AE and Loo JA: Nitrotyrosine-modified proteins and oxidative stress induced by diesel exhaust particles. Electrophoresis *26*: 280-292, 2005.

9 Xiao GG, Wang M, Li N, Loo JA and Nel AE: Use of proteomics to demonstrate a hierarchical oxidative stress response to diesel exhaust particle chemicals in a macrophage cell line. J Biol Chem *278*: 50781-50790, 2003.

10 Macek B, Waanders L, Olsen JV and Mann M: Top-down protein sequencing and MS^3 on a hybrid linear quadrupole ion trap-orbitrap mass spectrometer. Mol Cell Proteomics *5*: 949-58, 2006.

11 Liotta LA and Petricoin EF: Serum peptidome for cancer detection: spinning biological trash into diagnostic gold. J Clin Invest *116*: 26-30, 2006.

12 Li J, Zhao J, Yu X, Lange J, Kuerer H, Krishnamurthy S, Schilling E, Khan SA, Sukumar S and Chan DW: Identification of biomarkers for breast cancer in nipple aspiration and ductal lavage fluid. Clin Cancer Res *11*: 8312-8320, 2005.

13 Horgan GW: Sample size and replication in 2D gel electrophoresis studies J Proteome Res *6*: 2884-2887, 2007.

14 Moore RE, Young MK and Lee TD: Qscore: An algorithm for evaluating SEQUEST database search results. J Am Soc Mass Spectrom *13*: 378-386, 2002.

15 Liu X, Zhou B, Xue L, Qiu W, Shih J, Zheng S and Yen Y: Nuclear factor Y regulation and promoter transaction of human ribonucleotide reductase subunit M2 gene in a Gemcitabine resistant KB clone. Biochem Pharmacol *67*: 1499-1511, 2004.

16 Juhasz A, Frankel P, Cheng C, Rivera H, Vishwanath R, Chiu A, Margolin K, Yen Y, Newman EM, Synold T, Wilczynski S, Lenz HJ, Gandara D, Albain KS, Longmate J and Doroshow JH: Quantification of chemotherapeutic target gene mRNA expression in human breast cancer biopsies: comparison of real-time reverse transcription-PCR vs. relative quantification reverse transcription-PCR utilizing DNA sequencer analysis of PCR products. J Clin Laboratory Analysis *17*: 184-194, 2003.

17 Juhasz A, Vassilakos A, Chew HK, Gandara D and Yen Y: Analysis of ribonucleotide reductase M2 mRNA levels in patient samples after GTI-2040 antisense drug treatment. Oncology Rep *15*: 1299-1304, 2006.

18 Sadowski I, Ma J, Triezenberg S and Ptashne M: GAL4-VP16 is an unusually potent transcriptional activator. Nature *335*: 563-564, 1988.

19 Thadikkaran L, Siegenthaler MA, Crettaz D, Queloz PA, Schneider P and Tissot JD: Recent advances in blood-related proteomics Proteomics *5*: 3019-3034, 2005.

20 Petricoin EF, Ardekani AM, Hitt BA, Levine PJ, Fusaro VA, Steinberg SM, Mills GB, Simone C, Fishman DA, Kohn EC and Liotta LA: Use of proteomic patterns in serum to identify ovarian cancer. Lancet *359*: 572-577, 2002.

21 Bharti A, Ma PC, Maulik G, Singh R, Khan E, Skarin AT, Salgia R: Haptoglobin alpha-subunit and hepatocyte growth factor can potentially serve as serum tumor biomarkers in small cell lung cancer. Anticancer Res *24*: 1031-8, 2004.

22 Ahmed N, Barker G, Oliva KT, Hoffmann P, Riley C, Reeve S, Smith AI, Kemp BE, Quinn MA and Rice GE: Proteomic-based identification of haptoglobin-1 precursor as a novel circulating biomarker of ovarian cancer. Br J Cancer *9*: 129-40, 2004.

23 Ornstein DK, Rayford W, Fusaro VA, Conrads TP, Ross SJ, Hitt BA, Wiggins WW, Veenstra TD, Liotta LA and Petricoin EF 3rd: Serum proteomic profiling can discriminate prostate cancer from benign prostates in men with total prostate specific antigen levels between 2.5 and 15.0 ng/ml. J Urol *172*: 1302-5, 2004.

24 Xiao GG*, Recker RR and Deng HW: Recent advances in proteomics and cancer biomarker discovery Clinical Medicine: Oncology *2*: 1-10, 2008.