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Phospho-Proteomic Screen Identifies Potential Therapeutic Targets in Melanoma

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Abstract

Therapies directed against receptor tyrosine kinases are effective in many cancer subtypes, including lung and breast cancer. We used a phospho-proteomic platform to identify active receptor tyrosine kinases that might represent therapeutic targets in a panel of twenty-five melanoma cell strains. We detected activated receptors including TYRO3, AXL, MERTK, EPHB2, MET, IGF1R, EGFR, KIT, HER3, and HER4. Statistical analysis of receptor tyrosine kinase activation as well as ligand and receptor expression indicates that some receptors, such as FGFR3, may be activated via autocrine circuits. shRNA knockdown targeting three of the active kinases identified in the screen, AXL, HER3, and IGF1R, inhibited the proliferation of melanoma cells and knockdown of active AXL also reduced melanoma cell migration. The changes in cellular phenotype observed upon AXL knockdown appear to be modulated via the STAT3 signaling pathway, while the IGF1R-dependent alterations seem to be regulated by the AKT signaling pathway. Ultimately, this study identifies several novel targets for therapeutic intervention in melanoma.

Keywords

Melanoma; RTK; AXL; HER3; STAT3

Introduction

“Next generation” cancer therapies that target receptor tyrosine kinases (RTKs) have a major impact on the disease-free progression and survival of patients with breast cancer and non-small cell lung carcinoma (NSCLC). RTK inhibitors in clinical use include the antibodies Trastuzumab for *human epidermal growth factor receptor 2 (HER2)*-positive breast cancer and Cetuximab in *epidermal growth factor receptor (EGFR)*-positive colorectal cancer. Small molecule kinase inhibitors also target EGFR in NSCLC (Erlotinib), and KIT or platelet-derived growth factor (PDGFR) in gastro-intestinal stromal tumors (Imatinib).

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The incidence of melanoma has steadily risen in the United States over the past thirty years, yet individuals with late stage melanoma have a median survival time of only nine months (1–3). Imatinib has been used to treat melanomas with mutationally-activated KIT, while experimental studies demonstrate that targeting RTKs such as MET and insulin-like growth factor 1 receptor (IGF1R) may inhibit melanoma cell proliferation and survival (4–9). The use of RTK-directed therapeutics in melanoma has, however, been limited by the lack of available information about active RTKs in this disease.

In order to identify potential RTK therapeutic targets in melanoma, we surveyed the expression of all 58 human RTKs and their agonists in a panel of 24 low-passage melanoma cell strains and one commercially available melanoma cell line. The functional activation of 42 human RTKs was also examined and used to identify which RTKs are most frequently and intensely activated in melanoma. shRNA-mediated knockdown of AXL, HER3, and IGF1R, three of the RTKs identified in the screen, resulted in decreased melanoma cell proliferation. AXL knockdown also reduced melanoma cell migration and these cellular responses appear to be regulated by the signal transducer and activator of transcription 3 (STAT3) signaling pathway. These data identify new candidates for therapeutic intervention in melanoma.

Materials and Methods

Cell Culture

Yale University (YU) designated and WW165 melanoma cell strains were derived from primary and metastatic lesions as described (10). Melanoma tumors were excised as part of patient clinical care and were collected with the participants' informed written consent according to Health Insurance Portability and Accountability Act (HIPAA) regulations with the approval of the Yale Human Investigation Committee. Primary and metastatic melanoma cell strains were used prior to passage 20. Most melanoma cells were cultured in OptiMEM (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (basal medium). The WW165 primary melanoma and YUHEIK mucosal melanoma cells were grown in basal medium supplemented with 0.1 mM IBMX (3-isobutyl-1-methylxanthine, Sigma-Aldrich; St. Louis, MO). Normal human melanocytes from newborn foreskins (NBME1) and discarded adult skin (RMP32F) were grown in basal medium supplemented with 16 nM 12-O-tetradecanoyl phorbol-13-acetate (TPA), 0.1 mM 3-isobutyl-1-methylxanthine, 2.5 nM cholera toxin, 1 μ M Na₃VO₄, and 0.1 mM N₆,2'-O-dibutyryl-adenosine 3':5'-cyclic monophosphate (Sigma-Aldrich), termed TICVA (11). HEK293T cells from the American Type Culture Collection (ATCC, Rockville, MD) were grown in DMEM containing 10% FBS, 1% penicillin/streptomycin, and 1% HEPES. MDA-MB-231 cells, MDA-MB-453 cells, and BT474 cells (ATCC) were grown in RPMI containing 10% FBS and 1% penicillin/streptomycin.

Gene Expression Analysis

Whole genome gene expression analysis was based on data generated by the Yale SPORC in Skin Cancer (12). Briefly, NimbleGen human whole genome expression microarrays (array 2005-04-20_Human_60mer_ and array 2006-08-03_HG18_60mer) were used for hybridization of cDNA from normal melanocytes and melanoma cells at NimbleGen Systems Iceland LLC. Vínlandsleið 2–4, 113 Reykjavík, Iceland (currently Roche Applied Science, Basel, Switzerland) and by the Yale W.M. Keck Foundation Biotechnology Resource as described (12). The microarray data from melanoma cell lines (YUCAS, YUCOT, YUDOSO, YUGOE, YUHEF, YUHUY, YUKIM, YUKSI, YULAC, YUPLA, YUOB, YUROL, YUSIV, YUTICA, YUZOR, WW165 and YUHEIK) and four independent normal melanocytes cell cultures (NBME1 and RMP32F) were used to

determine a gene expression call (expressed/non expressed) and a mean RTK expression value. An expression call threshold (761.0) was determined using Stat4 library in R. Briefly, parameters of a bimodal normal distribution were estimated separating the gene expression intensity data into noise ($\mu_{\text{noise}} = 279.9$) and signal ($\mu_{\text{signal}} = 2360.6$). Genes were considered expressed if their observed intensity value (x) was greater than the $\mu_{\text{signal}} - 1\text{SD}_{\text{signal}}$ (i.e. $x > 761.0$). To assess correlation between RTK activation and ligand expression, we obtained a list of 473 known receptors ligands pairs from the Database of Ligand-Receptor Partners (DLRP) (13). The list was manually augmented with additional literature-derived receptor-ligand pairs. Within our data set 118 receptor-ligands pairs were used to correlate ligand expression (determined by NimbleGen array) with RTK phosphorylation (determined phospho-RTK Array).

RNA from snap-frozen melanoma tumors was extracted directly from frozen tumor blocks (YUCOT, YUSIV, and YUSTE) or after micro-dissection of snap-frozen tumors (YUDOSO and YULAC). RNA was isolated using the RNeasy Mini Kit from Qiagen (Valencia, CA) and reverse transcribed with the iScript cDNA Synthesis Kit from BioRad (Hercules, CA) using 0.8 μg of RNA per reaction. Universal TaqMan Master Mix (Applied Biosystems; Foster City, CA) was used to perform quantitative real-time PCR (qRT-PCR) using a 1 to 10 dilution of the resulting cDNA. The following primers were used following the manufacturer's protocols: AXL (Hs0024357_m1), HER3 (Hs00951455_m1), and GAPDH (Hs99999905_m1) (Applied Biosystems). Relative mRNA expression was determined with the delta Ct method using GAPDH as the reference gene. Gene expression values for tumor RNAs were normalized to a negative control. A single RNA preparation was analyzed for each micro-dissected tumor, and duplicates for each tumor block.

Cell Lysis, RTK Arrays, Immunoblotting, and Hierarchical Clustering

Where indicated, cells were starved in 0.1% serum prior to lysis, and pervanadate (50 μM final) was added for 20 minutes before lysis. Cells were lysed in NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris pH 7.4, 5 mM EDTA, 10% glycerol with complete EDTA-free protease inhibitor tablets [Roche] and Phosphatase Inhibitor Cocktails 1 and 2 [Sigma-Aldrich] added immediately before cell lysis). Cell lysates (250 μg) were analyzed with the Human Phospho-RTK Array Kit [R&D Systems (Minneapolis, MI)]; array maps at <http://www.rndsystems.com/pdf/ary001.pdf>. Protein samples were prepared for electrophoresis by addition of 5 $\times$ Laemmli sample buffer, and immunoblotting was performed on nitrocellulose membranes blocked in 5% milk in TBST (1.5 M NaCl, 0.2 M Tris-HCl, 0.05% Tween 20). Membranes were incubated with antibodies in 5% milk or 5% bovine serum albumin (BSA) overnight at 4°C: anti-pHER3, anti-pIGF1R, anti-IGF1R, anti-pKIT, anti-MER, anti-pMET, anti-MET, anti-pSTAT3, anti-STAT3, anti-pAKT, anti-AKT (Cell Signaling Technology; Boston, MA), anti-HER3, anti-GAPDH (Santa Cruz Biotechnology; Santa Cruz, CA), anti-TYRO3 (Abcam; Cambridge, MA), and anti-KIT (Dako; Carpinteria, CA). Secondary antibodies conjugated to horseradish peroxidase were used at a 1:10,000 dilution for one hour at room temperature prior to development (Thermo Scientific; Waltham, MA).

Non-supervised hierarchical clustering was performed using manually-determined phospho-array data values ranging from 0 (background) to 5 (maximum phosphorylation) (Tables 1–2). Clustering of RTK data was performed using Pearson correlation with complete linkage along sample and protein dimensions.

Immunoprecipitation

HER3 immunoprecipitation (IP) was performed with 1 mg of protein and 800 ng of HER3 antibody sc-7390 (Santa Cruz; Santa Cruz, CA); KIT IP with 300 μg protein and 1 μg of

A4502 (Dako); MET IP with 550 µg protein and 1 µg of MET antibody sc-10 (Santa Cruz); IGF1R IP with 500 µg protein and 100 ng of antibody 3027 (Cell Signaling Technology) at 4°C overnight. Then, 12 µL of a 50% slurry of protein A/G beads (Thermo Scientific; Waltham, MA) was added to each IP for 1 hour. Beads were washed twice with NP-40 lysis buffer and once with salt-Tris buffer (0.1 M NaCl, 10 mM Tris-HCl pH 7.4), then incubated at 100°C for 8 minutes in 2.5X Laemmli sample buffer.

shRNA and lentiviral infection

shRNA targeting AXL (RHS3979-9568950, AXL 1) (14) and (RHS3979-9568949, AXL 2), HER3 (RHS3979-9630819) (15), IGF1R (TRCN0000121300) (16), empty vector pLKO control (RHS4080) (Open Biosystems; Huntsville, AL), and a scrambled shRNA control (plasmid 1864) (Addgene; Cambridge, MA) were used. Lentivirus stocks were produced by cotransfecting 293T cells with pMD2.G and psPAX2 (Addgene) using FuGENE6 (Roche), with supernatants collected daily for three days and pooled (Addgene protocol). Experimental infections were performed at a multiplicity of infection of five.

Cell Proliferation

Cells were plated in duplicate clear-bottom 96-well plates at a density of 1,000 cells per well in 100 µL of OptiMEM. Approximately six hours after plating, cells were incubated with lentivirus in OptiMEM containing 4 µg/ml polybrene for 20 hours. Immediately following infection, a T₀ time point was taken by incubating one plate with CellTiter-Glo® (CTG) per the manufacturer's instructions (Promega; Madison, WI). Relative cell accumulation was calculated by dividing the value for the T₇₂ time-point by the value for the T₀ timepoint. Assays were repeated 4 times, with a representative plot shown for each.

Cell Migration

Cell lines were stably infected with scrambled shRNA or AXL shRNA and were seeded at 30,000 cells/well in 24-well plates with 8-micron filter inserts (BD Biosciences; Franklin Lakes, NJ) in OptiMEM with 0.1% FBS. OptiMEM with 5% FBS was used as a chemoattractant. Cell lines were analyzed in triplicate, with a representative graph chosen for each.

RESULTS

Transcriptional Profiling of Melanoma Cell Lines

We used transcription profiling to identify RTKs commonly expressed in melanoma cell lines and in normal melanocytes. Of the 58 human RTK genes, 43 RTKs were transcribed above a conservatively chosen expression threshold in at least one melanocyte culture, and 53 RTKs were above the threshold in at least one melanoma cell line (Supplementary Tables 1–2). Some RTKs, including *IGF1R*, *MET*, and *TYRO3*, were highly expressed in most melanomas and normal melanocytes, whereas *fibroblast growth receptor 2* (*FGFR2*), *EGFR*, and *ROS1* were rarely, if ever, detected above the expression threshold in either melanoma cells or melanocytes. *AXL* and *HER3* were expressed at higher levels in melanoma cells than in melanocytes, but *KIT* and *MERTK* expression was lower in melanoma cells compared to melanocytes (Supplementary Tables 1–2).

Survey of Active RTKs in Melanoma

Generally, the abundance of RTK mRNA does not predict functional activity: although RTKs such as HER2 and MET are frequently activated via gene amplification and over-expression, mutationally-activated RTKs can be potent oncogenes when expressed at low levels (17–18). Since tyrosine (Tyr) phosphorylation is indicative of the signaling activity of

RTKs (19), we surveyed receptor activation using phospho-arrays that capture 42 RTKs from cell lysates and are probed with anti-phospho-tyrosine (anti-PTyr). In order to enhance the endogenous PTyr signals, cells were also analyzed after treatment with pervanadate, a tyrosine phosphatase inhibitor. Pervanadate treatment significantly enhanced the cell lysate signals seen on the phospho-RTK arrays, but did not greatly alter the pattern of RTK activation (Figure 1A). Phospho-array controls included breast cancer BT474 and MDA-MB-453 cells, which are known to express active HER2, and MDA-MB-231 cells, which express little or no active HER3, but have substantial amounts of AXL (Table 1) (20–22).

As expected, RTK mRNA levels were only loosely correlated with receptor activation. For instance, *Colony-Stimulating Factor 1 Receptor (CSF1R)* mRNA levels were high in both melanoma cell lines and normal melanocytes, yet CSF1R was rarely phosphorylated (Tables 1–2, Supplementary Tables 1–2). The same trend was also observed for *EPHA2* in melanoma cell lines and *FGFR1* in normal melanocytes. Of the 42 RTKs analyzed via phospho-arrays, IGF1R, HER3, and MET were frequently activated and transcribed above the expression threshold in melanoma cell lines. Pervanadate treatment generally increased the number of RTKs activated and expressed above the threshold (Supplementary Tables 1–2). Phosphorylation of other RTKs was more variable in comparison to RNA expression, suggesting the importance of other modulators such as growth factors or active co-receptors.

RTK activation patterns were variable, even among cell lines with *BRAF* and *NRAS* activating mutations. Such mutations occur in 44% and 12%, respectively, of the melanoma cultures analyzed in this report. Examples of array variability are found in pervanadate-treated newborn melanocytes (NBMEs), which have higher levels of phosphorylated Macrophage Stimulating 1 protein Receptor (MST1R) than melanoma cell lines YUHEIK and YULAC (Figure 1B). Similarly, YUHEIK cells showed more activated EGFR than either newborn melanocytes or YULAC cells, while YULAC cells had lower activation of KIT and higher activation of IGF1R (Figure 1B). Despite these differences, the Insulin Receptor family (IR, IGF1-R), EGFR family (especially EGFR and HER3), MET family (MET and RON), and TAM family (TYRO3, AXL, and MERTK) were commonly activated in melanoma cultures (Tables 1–2). A ranking of receptor activation across all melanoma cell strains was determined by summing the phosphorylation intensities for each individual RTK (Supplementary Table 3). Interestingly, the 15 RTKs with the highest overall activation included receptors that had not been previously reported to be active in melanoma, including TYRO3, AXL, MERTK, and EPHB2 (Supplementary Table 3). RTKs such as EGFR, FGFR3, IR, IGF1R, and TYRO3 were frequently activated with and without pervanadate treatment while HER3, MET, and RON were more commonly detected after pervanadate treatment, perhaps reflecting faster turnover of phospho-tyrosine by endogenous phosphatases (Tables 1–2). The frequency of detection of phosphorylated EGFR and TYRO3 decreased with pervanadate treatment, possibly indicating a biological change such as heterologous desensitization through activation of other RTKs.

In both pervanadate-treated and untreated cultures, it was common to find multiple phosphorylated RTKs (Tables 1–2). Non-supervised clustering was used to evaluate possible co-associations (Supplementary Tables 4–5). Without pervanadate, there was some coassociation of IGF1R and TYRO3 phosphorylation, with a subgroup showing common activation with IR, FGFR3, and EPHB2 (Supplementary Table 4). With pervanadate treatment, activation of multiple RTKs across cell lines was more common (Supplementary Table 5). Recently, a related set of primary array data analyzing fifteen separate melanoma lines was reported. This data set identified active RTKs such as HER3, IGF1R, and TYRO3 in non-pervanadate-treated samples, but these results were not validated (23).

Validation of array data

We focused on validating TYRO3, MERTK, AXL, HER3, MET, KIT, and IGF1R because they were either among the most common or most novel active RTKs identified and because they represent attractive cancer therapeutic targets (Figure 1C–D). With the immunoblotting reagents available, we were able to determine the abundance of TYRO3 and MERTK, but could not assess their phosphorylation status for comparison with the phospho-array data. While high levels of phosphorylation in pervanadate-treated cells permitted analysis of total cell lysates, immunoprecipitated phosphorylated RTKs could also be detected in untreated cells. The pattern of receptor activation in the melanoma cell lines analyzed in Figure 1 did not vary substantially with pervanadate treatment (Figure 1C–D). We considered the immunoblots more accurate in the instances where the arrays did not correlate with the immunoblots. Hence, the RTK arrays provided a useful, but imprecise, indication of which RTKs are active in a given cell line.

RTK activation in melanoma

To identify receptors that might be activated through autocrine circuits or over-expression, we compared the phosphorylation levels determined by phospho-array analysis to the transcriptional levels of the RTKs and their respective ligands. In untreated cell lines, a few RTK phosphorylations correlated with ligand mRNA expression including FGFR3::FGF13, AXL::GAS6, and HER4 with assorted ligands (Supplementary Table 6). Similarly, in pervanadate-treated cells, moderate correlations were found between RTK phosphorylation and ligand expression for AXL::GAS6, EPHA2::EFNA3, and FGFR3::FGF13. In both datasets, receptor phosphorylation was moderately associated with RTK mRNA expression for AXL, while ERBB4 and EGFR also showed correlations in untreated cells and KIT mRNA expression was correlated with KIT activation in pervanadate treated samples (Supplementary Table 6).

The small number of cases for which receptor phosphorylations were identified in association with high ligand expression represent candidates for autocrine loops. Here, the limited sensitivity of the RTK arrays coupled with the possible chronic downregulation of ligand-activated RTKs means that there may be many false negatives. Additionally, elevated expression can move the designated RTK above phospho-array threshold for detection, which may skew the results of our screen. The co-associations of RTK phosphorylation with high receptor mRNA levels may, however, identify cases for which RTK overexpression contributes to receptor activation.

The strongest correlations were found in comparison of receptor and ligand mRNA expression levels. For a subset of RTK/ligand pairs, including FGFR3 with several of its ligands, PDGFRβ::PDGFβ, AXL::GAS6, and ERBB4::NRG3, there were strong associations of mRNA levels (Supplementary Table 6). These RTK/ligand pairs represent candidates for autocrine activation, although for some pairs subcellular compartmentalization or the need for proteolytic activation of pro-hormones may limit RTK activity.

RTK knockdown influences melanoma cell proliferation, migration, and signaling

The phospho-array survey identified IGF1R as active in most of the melanoma cell lines (Tables 1–2). Since cells were maintained in medium supplemented with serum, which is rich in ligand IGF1, we explored whether IGF1R phosphorylation was affected by serum concentration. Three melanoma cell lines were serum-starved for a period of 24–48 hours and immunoblotting was used to measure the presence of total and phosphorylated IGF1R (Figure 2A). The persistence of IGF1R phosphorylation indicates that serum IGF1 is not the primary factor influencing IGF1R activation.

The high prevalence of IGF1R activation in our panel of melanoma cell lines suggested that it might be functionally important. Four melanoma cell lines characterized by phosphorylated IGF1R were infected with empty vector (pLKO), scrambled shRNA (SC), or shRNA against IGF1R (Tables 1–2, Figure 2B). IGF1R knockdown corresponded with decreased cell proliferation and a reduction in AKT signaling, in accordance with earlier studies (6) (Figure 2B–C).

AXL and HER3 were selected for further validation of the data generated by the phospho-array screen. We focused on these two receptors because they are relatively unexamined in the context of melanoma pathogenesis and because TAM family members are poorly characterized in cancers in general. Lentiviral vectors encoding shRNA directed against AXL or HER3 were used to knock down the respective receptors singly and in combination (Figure 3A). The growth of YURIF cells, which do not express AXL or HER3 (Tables 1–2, Figure 1C–D), was not affected by lentiviral-mediated knockdown of either RTK (Figure 3B). In contrast, knockdown of AXL or HER3 strongly inhibited proliferation of YUDOSO cells (Figure 3B), which express active AXL and HER3 (Figure 1C–D). A milder reduction in proliferation was obtained by targeting active HER3 in YUROL cells (Tables 1–2, Figure 3B). YUKSI cells, which express AXL but have low AXL activity (Tables 1–2, Figure 1C–D, Figure 3A), displayed only slight reductions in proliferation and migration upon AXL knockdown (Figure 3D–E). These alterations in proliferation and migration correlated with a slight reduction in phospho-STAT3 (Figure 3A). Knockdown of active AXL in YUSIV, YUSTE, and YUTICA melanoma cells evoked a more pronounced decrease in cell proliferation, cell migration, and STAT3 phosphorylation (Tables 1–2, Figure 3A, D–E). Sporadic reductions in phospho-AKT were also observed upon AXL knockdown (data not shown). Further validation of these effects was obtained by targeting AXL using a second shRNA in four melanoma cell lines (Supplementary Figure 1). These results support the biological relevance of AXL and HER3 in melanoma cells and substantiate the importance of targeting activated receptors.

Melanoma cell lines predict presence of RTKs in tumors

To verify that expression of the RTKs identified in our screen is not a cell culture artifact, snap-frozen tissues derived from the same original tumors were analyzed via qRT-PCR. *AXL* mRNA was identified by transcriptional profiling in YUDOSO, YUSIV, and YUSTE, but not in YUCOT melanoma cells (Table 3). *AXL* mRNA and protein were variably detected in YULAC cells (Figure 1C–D, Table 3). qRT-PCR analysis of frozen tumor tissues revealed that YUDOSO, YUSIV, YUSTE, and YULAC tumors all express *AXL*, but the YUCOT tumor did not (Figure 4A). Similarly, *HER3* mRNA was detected by transcriptional profiling of the YUCOT, YULAC, YUDOSO, YUSIV, and YUSTE melanoma cell strains as well as by qRT-PCR analysis of tissue from the corresponding tumors (Table 3, Figure 4B).

As expected, there was no direct correlation between the activity of an RTK in a cell line (Tables 1–2) and the presence of mRNA in the corresponding tumor (Figure 4A–B). Nonetheless, activated AXL and HER3 were only detected in melanoma cell lines for which the tumors expressed the corresponding receptors. The differences in rank expression of the RTKs in tumors versus cell lines may reflect tumor to cell strain differences, tumor heterogeneity, or differential infiltration by non-tumor cells in the tumor micro-environment.

Discussion

We report here the first broad survey of functionally activated RTKs in a panel of early-passage melanoma cell strains. This survey represents one facet of an integrated analysis of a reference set of melanoma cell lines and corresponding tumor tissue that will eventually

encompass transcription profiling, epigenetic analysis, copy number analysis, transcriptome and exome resequencing, and drug response profiling. We found that the insulin receptor family (IGF1R, IR), MET family (MET, MST1R), EGFR family (EGFR, HER3, HER4), and TAM family (TYRO3, AXL, MERTK) were commonly activated in melanoma (Tables 1–2).

IGF1R was frequently and strongly activated in our panel of cell strains, and this activation did not appear to be serum dependent (Tables 1–2, Figure 2A). A previous report demonstrated that knockdown of IGF1R can greatly reduce melanoma cell growth and that targeting IGF1R reduced signaling through both the AKT and the mitogen-activated protein kinase (MAPK) pathways (6). Our data also support the notion that knockdown of IGF1R may reduce cell growth in an AKT-dependent manner (Figure 2). Activation of IGF1R promotes liver metastases in uveal melanoma and activated IGF1R, along with activated HER3, is known to contribute to Herceptin resistance in breast cancer (24–27). Signaling through IGF1R also compensates for therapeutic inhibition of mTOR in breast and prostate cancer cells (28). It is possible that activated IGF1R might serve an analogous role in melanoma and it will therefore be important to determine the mechanism of IGF1R activation in melanoma.

A recent study demonstrated that HER4 may be somatically mutated in up to 19% of melanomas, and our survey reveals frequent activations of the EGFR family members in melanoma (29). While this work was in progress, elevated HER3 expression and activation was reported in melanoma cells relative to normal melanocytes, with knockdown of HER3 reducing melanoma cell proliferation, migration, and invasion (30–31). Reduced HER3 expression was also correlated with reduced phospho-AKT and increased p27 expression (31). The findings presented here are consistent with these reports, since our melanoma cell strains frequently show higher HER3 activation and mRNA than melanocytes (Tables 1–2; Supplementary Tables 1–2) and since HER3 knockdown reduces melanoma cell proliferation (Figure 3). It has been proposed that activation of HER3 by NRG1 contributes to melanoma development and progression (30), but we did not identify a correlation between HER3 activation and the expression of ligands NRG1 or NRG2 (Supplementary Table 6). One explanation may be that NRG1 is important in early stages of melanoma but its expression does not persist in later stages; this is consistent with the reported higher frequency of activated HER3 in primary compared to metastatic melanomas (30). The phospho-RTK array analysis also reveals co-activation of MET with EGFR family members such as HER3 (Tables 1–2). Since coordinate activation of MET with ERBBs often modulates resistance to EGFR inhibitors in non-small cell lung carcinoma, combined inhibition of MET and ERBBs may be beneficial for a subset of melanomas as well (32).

The TAM receptor family is well-studied in inflammation but is not yet completely characterized in carcinogenesis. Previous work shows that *AXL* mRNA is elevated in melanomas relative to normal melanocytes, in agreement with our transcriptional data (Supplementary Tables 1–2) (33). Although *TYRO3* and *MERTK* were reported to be preferentially expressed in melanomas, we found that *TYRO3* is highly expressed in both melanocytes and melanomas and that *MERTK* expression is, on average, lower in melanoma cells compared to normal melanocytes (Supplementary Tables 1–2) (33–35). Interestingly, the phospho-arrays revealed that at least one member of the TAM family was activated in the majority of the melanoma cell lines, even without pervanadate treatment (Tables 1–2). Further, activation of AXL and MERTK may have some degree of mutual exclusivity, since AXL and MERTK activation rarely occurred concurrently in the melanoma cell lines.

Suppression of TYRO3 reduces melanoma cell proliferation, sensitizes cells to chemotherapeutics, decreases colony formation, and reduces tumor formation and growth

(34). Further, AXL is required for GAS6-dependent uveal melanoma cell survival (36). In our study, knockdown of activated AXL noticeably affects cell proliferation in a primary cutaneous melanoma line (YUDOSO), three metastatic cutaneous lines (YUGEN8, YUSIV, YUSTE), and two cutaneous lines that had metastasized to the lung (YUHEF, YUTICA) (Figure 3, Supplementary Figure 1). Targeting AXL in a subset of these cell lines also reduces cell migration (Figure 3). It appears that this decrease in migration and proliferation correlates with a reduction in STAT3 signaling (Figure 3, Supplementary Figure 1). Thus, AXL signaling may contribute to the constitutive activation of STAT3 that is frequently observed in human melanoma cell lines and tumors (37).

Some of the RTKs identified by our screen (IGF1R, MET, and KIT) have been previously described in melanomas. Others, including HER3, HER4, members of the TAM family, EPHB2, and MST1R, have either not been reported as active, or are poorly characterized in this disease. These findings have therapeutic implications because some of the receptors identified in the melanoma cell lines, specifically AXL and HER3, were found in the corresponding tumor tissue (Table 3, Figure 4). Targeting AXL in a cell line that either does not express phosphorylated AXL (YUKSI) or in a cell line that does not express the receptor at all (YURIF) had little to no effect on cell behavior (Figure 3). Interestingly, targeting the activated receptors appears to be effective regardless of *NRAS* and *BRAF* mutation status. Both IGF1R and AXL knockdown alters cell behavior in *BRAF*/*NRAS* wild-type cells (YUHEF, YUSIV), *NRAS* mutant cells (YUDOSO, YUTICA), and *BRAF* mutant (YUGEN8, YUKSI, YULAC, YUSTE) melanoma cell lines. Although HER3 knockdown was not examined in a *BRAF* mutant line, it is effective in *NRAS* (YUDOSO) mutant and wild-type (YUROL) cell lines (Figure 3). These findings are important because expression and activation of RTKs was largely independent of mutation status, and because activation of non-mutated RTKs is a common mechanism of resistance in tumor cells attacked with RTK or pathway-directed therapeutics (38). In fact, in a non-biased survey of protein kinase cDNAs for the ability to promote melanoma cell resistance to the activated *BRAF* inhibitor PLX4720, AXL was identified as the RTK best able to rescue cell proliferation (39). Another study identified upregulation and activation of PDGFR β receptor as a route to resistance in *BRAF* mutated melanoma treated with PLX4032 (40). Perhaps co-expression of ligand-receptor conjugate pairs, as we found for several RTKs including PDGFR β , foreshadows routes to resistance.

These findings document variability in RTK expression and activation among melanomas. Identification of cancer drivers by gene mutation analysis is attractive since the technology is accurate and sensitive and since mutations in protein kinases and other signaling proteins can have strong predictive value. Nonetheless, carcinogenic alterations in RTKs and signaling pathways are also induced by changes in expression, sub-cellular localization, and proteolysis of ligands. Since RTKs are mainly regulated through protein-protein interactions and protein modifications, functional analyses that measure protein changes continue to provide important insights into cancer at the discovery and treatment levels. For instance, one of the early indications of ALK activation in a subset of lung cancers was derived from a mass spectrometry survey of lung cancer tumors and cell lines (41). The fact that *HER3*, *AXL*, and *IGF1R* knockdown was effective regardless of *NRAS* and *BRAF* status suggests that inhibition of RTKs may be valuable in combination with *RAF* or *MEK* inhibitors (Figure 3). The RTKs identified in this survey may provide new therapeutic opportunities for driver inhibition while improving our ability to prevent therapeutic resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Analysis of RTK phosphorylation. A, Comparison of YUDOSO cell lysates incubated in the absence (left) or presence (right) of pervanadate (PV) prior to lysis. Each pair of vertical spots represents one receptor. Paired spots in the four corners are positive PTyr controls, and appear darker at left because of the longer film exposure time required for the lower signals without pervanadate treatment. B, Comparison of RTK activation profiles among pervanadate-treated NBME1, YUHEIK, and YULAC cells. Spots representing specific receptors are boxed in each panel. C–D, Lysates from non-treated (panel C) and pervanadate-treated (panel D) melanoma cell lines were analyzed by immunoblotting for total and Tyr-phosphorylated receptors. Total cell lysates (TCL) were analyzed by immunoblotting with anti-RTK and anti-phospho-RTK antibodies. RTK immunoprecipitates (IP) were analyzed with anti-phospho-RTK, with control immunoglobulin (IgG) IP as negative control. All immunoblots have been cropped.

Figure 2. IGF1R activation in melanoma cell signaling and proliferation. A, Melanoma cell lines were maintained in 0.1% FBS or 5% FBS for 24 or 48 hours prior to lysis. Lysates were analyzed for phospho-IGF1R, IGF1R, and the GAPDH loading control. Three biological replicates are shown. Image J analysis was used to quantify phospho-IGF1R relative to GAPDH for each sample. Average ratios $\pm$ one standard deviation for the samples shown, from left to right, are as follows: 0.78 ± 0.29 , 1.1 ± 0.08 , 0.79 ± 0.29 , 0.81 ± 0.16 , 0.76 ± 0.07 , 0.76 ± 0.01 , 0.77 ± 0.09 , 0.69 ± 0.24 , 0.81 ± 0.26 , 0.81 ± 0.21 , 0.50 ± 0.01 , 0.47 ± 0.26 . B, Expression of phospho-IGF1R, IGF1R, phospho-AKT, and AKT in melanoma cells infected with lentiviruses encoding backbone only (pLKO), scrambled shRNA (SC), or shRNA for IGF1R. All immunoblots have been cropped to improve readability. C, Growth of lentivirus-infected cells assessed over a 72-hour period using the CellTiter-Glo Luminescent Cell Viability Assay.

Figure 3. Impact of AXL and HER3 knockdown on cell proliferation, migration, and signaling. A, Expression of phospho-AXL, AXL, HER3, phospho-STAT3, and STAT3 with GAPDH loading control in melanoma cells infected with empty backbone (pLKO), scrambled shRNA (SC), or shRNA for AXL and/or HER3. Immunoblots have been cropped. B–D, Growth of lentivirus-infected cells assessed over a 72-hour period using the CellTiter-Glo Luminescent Cell Viability Assay. Melanoma cells are grouped based on the viruses used; cell lines in the same panel were generally analyzed in different experiments. E, Migration of melanoma cells infected with control (SC) or AXL shRNA lentivirus relative to mock-infected cells. AXL knockdown was achieved using “AXL 1” shRNA sequence.

Figure 4. Analysis of RTK mRNA in tumor samples. A–B, Relative expression of *AXL* and *ERBB3* mRNA from tumors. *AXL* and *HER3* expression were measured by qRT-PCR and normalized to GAPDH, and the resulting expression is shown relative to negative controls for *AXL* (YURIF) and *HER3* (MB231).

Table 1

Survey of tyrosine phosphorylated receptors by RTK capture array analysis in untreated melanoma cell lines. Summary of receptor activations in breast cancer cell lines, melanoma cell lines, and newborn melanocytes (NBME). Signal intensity was visually scored on a scale from 0 (background) to 5 (most intense). Melanoma cells are grouped by *NRAS/BRAF* status.

	Breast Cancer			Melanomas and Normal Melanocytes (NBME)																										
	ND*			WT <i>BRAF/INRAS</i>														<i>NRas</i>			<i>BRaf</i>									
																		Q61R	Q61R	Q61K	V600E	V600E	V600E	V600E	V600E	V600E	V600K	V600K	V600K	V600K
	BT474	MB231	MB453	NBME	YUGOE	YUHEF	YUHEIK	YUHOIN	YUKIM	YUNIGE	YUPLA	YUROB	YUROL	YUSIV	YUZOR	YUCAS	YUTICA	YUDOSC	YUCOT	YUGEN8	YUHUY	YUSAC2	YUSIK	YUSTE	YUKSI	YULAC	YUMAC	YURIF	WW165	
EGFR	5	0	5	0	1	5	3	0	2	1	1	3	0	0	1	2	0	2	0	3	2	0	1	1	5	3	1	1	3	
HER2	4	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	
HER3	3	0	0	0	0	3	0	0	0	1	0	0	4	0	0	2	2	2	0	1	0	0	2	0	1	0	0	0	0	
HER4	0	0	0	0	0	1	0	0	3	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	5	0	1	1	0	
FGFR1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
FGFR2a	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	
FGFR3	0	0	0	1	1	1	3	0	5	0	0	1	0	0	0	2	2	3	3	4	3	0	2	1	0	0	0	0	0	
FGFR4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
InsulinR	0	0	0	0	3	0	3	1	5	0	0	0	0	0	0	4	3	3	0	3	3	3	4	3	0	0	0	1	3	
IGF1R	0	0	0	3	5	5	3	1	5	2	3	5	4	5	5	5	5	5	0	5	5	3	5	5	3	5	5	5	5	
AXL	4	3	3	0	0	0	0	3	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	
TYRO3	1	0	0	5	3	0	3	4	4	2	1	5	5	0	5	4	1	0	0	4	3	3	4	5	3	5	5	5	5	
MERTK	1	0	0	1	0	1	0	0	3	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	
MET	0	0	1	1	0	1	0	0	4	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	3	0	
MST1R	0	0	0	0	1	1	3	0	0	0	0	1	1	0	0	0	2	1	0	1	2	0	0	0	0	1	0	0	1	
PDGFRa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
PDGFRb	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	
KIT	0	0	0	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	
FLT-3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
M-CSFR	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	
RET	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	4	0	0	0	3	0	
ROR1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	1	0	0	0	0	0	1	0	0	0	
ROR2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
TIE-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	
TIE-2	1	0	0	0	0	0	3	0	2	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	
TRKA	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	
TRKB	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
TRKC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
VEGFR1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	
VEGFR2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
VEGFR3	0	0	0	0	0	1	0	0	3	0	0	0	0	0	0	0	3	3	0	0	0	0	0	0	0	0	0	0	3	0
MUSK	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
EPHA1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
EPHA2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
EPHA3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
EPHA4	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
EPHA6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
EPHA7	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
EPHB1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	
EPHB2	0	0	0	0	1	1	2	1	2	0	0	1	1	0	1	2	3	3	2	3	3	0	0	1	0	3	1	0	0	
EPHB4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
EPHB6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

* *BRAF* and *NRAS* not determined (ND) for breast cancer cell lines.

Table 2

Survey of tyrosine phosphorylated receptors by RTK capture array analysis in pervanadate (PV)-treated melanoma cell lines. Summary of RTK activation in melanoma cell lines and newborn melanocytes (NBMEs) scored as described in legend to Table 1.

With PV	Melanomas and Normal Melanocytes (NBME _L)																										
	WT <i>BRaf</i> / <i>NRas</i>												<i>NRas</i>			<i>Braf</i>											
													Q61R	Q61R	Q61K	V600E	V600E	V600E	V600E	V600E	V600E	V600K	V600K	V600K	V600K		
	NBME	YUGOE	YUHEF	YUHEIK	YUHOIN	YUKIM	YUNIGE	YUPLA	YUROB	YUROL	YUSIV	YUZOR	YUCAS	YUTICA	YUDOSO	YUCOT	YUGEN8	YUHUY	YUSAC2	YUSIK	YUSTE	YUKSI	YULAC	YUMAC	YURIF	WW165	
EGFR	0	0	5	3	5	0	0	5	3	0	5	5	2	0	0	0	0	0	0	0	0	0	0	0	0	0	
HER2	1	1	0	3	5	0	4	1	1	1	0	0	1	4	4	1	3	4	0	1	0	0	0	0	0	1	3
HER3	1	5	5	5	0	5	5	0	5	5	0	5	5	5	5	5	5	5	5	5	5	5	5	5	0	5	
HER4	3	1	4	0	5	4	1	0	3	3	4	0	1	1	3	1	4	3	1	1	4	5	0	1	1	0	
FGFR1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	
FGFR2a	3	0	0	0	0	0	0	0	0	0	0	0	0	2	2	0	0	0	0	0	0	0	0	0	0	0	
FGFR3	4	1	3	0	4	4	1	1	4	3	0	1	4	5	3	5	5	5	1	3	3	1	1	0	3	1	
FGFR4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
InsulinR	4	5	2	5	0	3	0	1	1	1	0	3	4	3	3	4	4	3	5	3	3	0	0	1	0	1	
IGF1R	5	5	5	5	4	3	3	5	4	4	3	5	5	5	5	4	5	5	5	5	5	5	5	5	3	3	
AXL	0	0	3	0	4	0	0	5	0	0	1	1	1	3	3	0	1	0	1	0	1	1	0	1	0	1	
TYRO3	0	0	0	0	0	0	1	0	0	0	0	1	1	3	2	0	3	5	3	1	1	3	3	1	0	1	
MERTK	1	0	3	0	0	3	2	0	0	1	0	0	1	1	0	2	3	3	1	1	0	0	3	1	0	3	
MET	4	5	5	5	5	2	3	3	0	3	3	0	5	3	0	2	5	0	0	1	3	0	3	1	5	3	
MST1R	5	1	5	5	3	4	0	0	3	3	0	1	1	3	5	4	3	5	1	3	1	0	3	3	1	3	
PDGFRa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
PDGFRb	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
KIT	5	0	3	5	0	1	0	0	0	0	0	0	0	0	0	0	0	5	0	1	0	0	3	5	5	3	
FLT-3	3	1	3	0	1	3	0	1	1	3	0	1	3	3	3	2	3	3	3	1	1	1	1	3	1	1	
M-CSFR	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
RET	0	1	2	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	0	1	
ROR1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
ROR2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
TIE-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	
TIE-2	0	0	3	0	0	2	0	0	0	1	0	0	0	1	3	2	1	0	0	0	0	0	0	0	0	1	
TRKA	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	
TRKB	0	0	2	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	
TRKC	0	0	2	0	0	0	0	0	0	0	0	0	0	0	3	1	0	0	1	0	0	0	0	0	0	0	
VEGFR1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	
VEGFR2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
VEGFR3	3	1	3	0	1	3	0	1	1	3	0	1	3	3	5	2	3	3	1	1	1	1	1	3	1	1	
MUSK	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	
EPHA1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
EPHA2	0	0	0	0	3	2	0	1	0	0	0	3	0	0	0	0	0	0	0	0	1	0	0	0	0	0	
EPHA3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
EPHA4	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	2	3	3	1	0	0	0	1	0	1	
EPHA6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
EPHA7	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	
EPHB1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
EPHB2	1	1	2	0	1	0	0	0	0	0	0	0	2	0	0	0	1	0	1	0	0	0	3	1	0	1	
EPHB4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
EPHB6	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

Table 3

Receptor expression and activation in cell lines. Whole genome RNA expression values determined for AXL (NM_021913) and *ERBB3* (*HER3*) (NM_001982) in melanoma cell lines. Receptor activation values are from RTK capture array analyses of pervanadate-treated (+PV) and non-treated (−PV) cells.

Cell Line	Activation of AXL on Array		Transcriptional Expression of AXL	Activation of HER3 on Array		Transcriptional Expression of <i>ERBB3</i>
	− PV	+ PV		− PV	+ PV	
NBMEL	0	0	842	0	1	943
YUCOT	0	0	466	0	5	15,164
YUDOSO	0	3	2,500	2	5	21,188
YULAC	0	0	425	0	5	12,623
YUSIV	0	1	8,327	0	0	9,717
YUSTE	0	1	8,351	0	5	10,251