Identification of Common and Distinctive Mechanisms of Resistance to Different Anti-IGF-IR Agents in Ewing's Sarcoma

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IGF system contributes significantly to many human malignancies. Targeting IGF-I receptor (IGF-IR) has been reported to be active against several tumors, but particular efficacy was observed only against a minority of Ewing's sarcoma patients. Identification of mechanisms of acquired resistance to anti-IGF-IR agents is mandatory to individualize their use in clinics and optimize cure costs. In this study, we compared gene expression profiles of cells made resistant with three different anti-IGF-IR drugs (human antibodies AVE1642, Figitumumab, or tyrosine kinase inhibitor NVP-AEW541) to highlight common and distinctive mechanisms of resistance. Among common mechanisms, we identified two molecular signatures that distinguish sensitive from resistant cells. Annotation analysis indicated some common altered pathways, such as insulin signaling, MAPK pathway, endocytosis, and modulation of some members of the interferon-induced transmembrane protein family. Among distinctive pathways/processes, resistance to human antibodies involves mainly genes regulating neural differentiation and angiogenesis, whereas resistance to NVP-AEW541 is mainly associated with alterations in genes concerning inflammation and antigen presentation. Evaluation of the common altered pathways indicated that resistant cells seem to maintain intact the IGF-IR internalization/degradation route of sensitive cells but constantly down-regulated its expression. In resistant cells, the loss of proliferative stimulus, normally sustained by IGF-I/IGF-IR autocrine loop in Ewing's sarcoma cells, is compensated by transcriptional up-regulation of IGF-II and insulin receptor-A; this signaling seems to favor the MAPK pathway over the v-akt murine thymoma viral oncogene homolog 1 pathway. Overall, complexity of IGF system requires analytical evaluation of its components to select those patients that may really benefit from this targeted therapy and support the idea of cotargeting IGF-IR and insulin receptor-A to increase the efficacy. (***Molecular Endocrinology*** **26: 1603–1616, 2012)**

The IGF system contributes significantly to many human malignancies. Both *in vitro* and *in vivo* studies have clearly implicate IGF and their receptors in the regulation of crucial processes, such as anchorage-independent growth, migration, metastasis, and resistance to several anticancer agents. The IGF system is composed of

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Abbreviations: AKT, v-akt murine thymoma viral oncogene homolog 1; DXR, doxorubicin; EWS, Ewing's sarcoma; FBS, fetal bovine serum; FC, fold change; FDR, false discovery rate; HAb, human antibody; IFITM, interferon-induced transmembrane protein; IGF-IR, IGF-I receptor; IMDM, Iscove's modified Dulbecco's medium; IR, insulin receptor; ISG, interferon-stimulated gene; LAMP-1, lysosomal-associated membrane protein 1; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide); Nanog, Nanog homeobox; OCT, octamer-binding transcription factor; siRNA, small interfering RNA; SLC, solute carrier family; TKI, tyrosine kinase inhibitor; VCR, vincristine.

multiple receptors and ligands (for a review, see Refs. 1, 2). Briefly, there are three ligands (IGF-I, IGF-II, and insulin), four receptors, at least six high-affinity binding proteins and binding protein proteases. The IGF-I receptor (IGF-IR) shows high level of sequence identity, particularly within the intracellular kinase domain (84% of homology), to the insulin receptor (IR). This homology is important to determine the overlapping but different biological effects of IGF-IR and IR. Two isoforms of IR are generated by alternative splicing of exon 11, giving rise to the exon 11+ (IR-B) and exon 11− (IR-A), which lacks the 12 amino acids of exon 11. The two isoforms are differentially expressed during development, with IR-A prevalent in fetal tissues and IR-B in adult tissues, particularly liver, muscle, and adipose tissue. Insulin and IGF bind with high affinity to their cognate receptor and with lower affinity to the noncognate receptor, with the exception of IGF-II, which also binds IR-A with high affinity (3). Although both IR and IGF-IR similarly activate phosphatidylinositol 3 kinase and MAPK pathways (4, 5), subtle differences exist in the recruitment of certain docking proteins and intracellular mediators. These differences are the basis for the predominant metabolic effects elicited by IR activation and the predominant mitogenic, transforming, and nonmetabolic effects elicited by IGF-IR activation (6). Recent findings have, however, added at least two layers of complexity in IGF system regulation. The first level of complexity is related to the documented role of IR in mitogenesis and cell motility (for detailed review, see Ref. 7). The second is due to the existence of hybrids between IGF-IR and IR. These receptors, especially those containing the fetal isoform IR-A prevalent in tumors (3), have the interesting biological characteristic to be activated by both IGF and insulin and also to activate both IR and IGF-IR β-subunits, thus overcoming specificity in intracellular signaling pathways. Cross talk between IGF-IR and IR appears more frequent and more relevant for cancer development and progression than we previously thought. This important fact has implications for therapy. Currently, most therapeutic agents, human antibodies (HAb) or tyrosine kinase inhibitors (TKI), have been designed to specifically target IGF-IR while sparing IR, based on the concern that cotargeting IR would have lead to unacceptable toxicity. However, phase I–III clinical studies with anti-IGF-IR drugs have clearly indicated modest toxic effects, with mild and reversible hyperglycemia as the most common toxicity, but limited effectiveness. Particularly in Ewing's sarcoma (EWS), despite the presence of the target in all tumors and ample preclinical evidence supporting the potential value of anti-IGF-IR agents, less than 10% of cases extraordinary responded to this therapy (8, 9). We and others have recently provided evidences for a compensatory role of IR-A when IGF-1R is disrupted (10–12), indicating the relationship between these two receptors as one mechanism responsible of acquired and intrinsic resistance to selective anti-IGF-IR therapy. However, other mechanisms have been described in different tumors (13–16), and further studies are clearly necessary to better define patients that may really benefit from an anti-IGF-IR therapy as well as to rationalize the use of this targeted therapy in combination treatments.

In this study, we compared resistance mechanisms for two specific anti-IGF-IR HAb, the AVE1642 or the CP-751,871 (Figitumumab), and the anti-IGF-IR TKI NVP-AEW541. Three EWS cell variants specifically resistant to each drug have been genetically and molecularly analyzed to identify common as well as distinct mechanisms of acquired resistance, to provide insights to be used for reversal or prevention of resistance. In addition, we identified a restricted genetic signature highly associated with anti-IGF-IR resistance that may find application in appropriate selection of patients.

Materials and Methods

Drugs

The anti-IGF-IR drugs were kindly provided by: Immunogen (AVE1642 HAb; Waltham, AM), Pfizer (CP-751,871/Figitumumab; San Diego, CA), and Novartis (NVP-AEW541; Basel, Switzerland).

Establishment of resistant cells

The EWS TC-71 cell line was recently authenticated (17). Cells resistant to the anti-IGF-IR HAb AVE1642 and to the TKI NVP-AEW541 were obtained from TC-71 cell line by exposure to increasing concentrations of the anti-IGF-IR HAb AVE1642 (up to 10 μg/ml) or to the TKI NVP-AEW541 (up to 5 μM) for 6 months, as recently described and characterized (11). Similarly, we obtained and here described a new cell line variant resistant to 100 μg/ml of anti-IGF-IR HAb Figitumumab (CP-751,871) (18). Resistant variants were referred here as TC/AVE or TC/AEW or TC/CP. All these cell variants were tested for mycoplasma contamination every 3 months (last control, November 2011) by PCR Mycoplasma detection set (TaKaRa Bio, Inc., Shiga, Japan). In experimental conditions, cells were maintained in standard medium [Iscove's modified Dulbecco's medium (IMDM), plus 10% fetal bovine serum (FBS)] lacking selecting agents for at least 48 h, to avoid effects of direct exposure to HAb or TKI.

In vitro assays

To assess cell growth, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Roche, Indianapolis, IN) was used according to manufacturer's instructions. Cells were plated into 96-well plates (2500 cells/well) in IMDM plus 10% FBS. After 24 h, various concentrations of AVE1642 (1–100 ng/ml), and vincristine (VCR) (0.01–50 μg/ml), NVP-

AEW541 (0.03–5 μM), Figitumumab (0.5–500 μg/ml), doxorubicin (DXR) (0.1–3 ng/ml) were added and cells exposed to these drugs for up to 72 h. Anchorage-independent growth was determined after seeding 1000 cells/dish in 0.33% agarose (SeaPlaque; FMC BioProducts, Rockland, ME) with a 0.5% agarose underlay (19). IFITM1 expression was transiently silenced by a pool of three target-specific small interfering RNA (siRNA) from Santa Cruz Biotechnology, Inc. (San Diego, CA). TC/AVE or TC/AEW were pretreated with IFITM1 siRNA (50 nM) for 24 h and then exposed to AVE1642 (0.1–10 μg/ml) or to NVP-AEW541 (0.5–5 μM) for 48 h. On-TargetPlus NonTargeting Pool (Dharmacon, Chicago, IL) was used as control.

Reverse transcription-polymerase chain reaction

Total RNA (2 mg) was extracted by TRIzol and reverse transcribed by ThermoScript RT (Invitrogen, Carlsbad, CA) and Oligo dT primers (Applied Biosystems, Carlsbad, CA). Quantitative real-time PCR was performed on ABI Prism 7900 (Applied Biosystems) using predesigned TaqMan gene expression Assay (IGF binding protein-3, Hs00426287_m1) and SYBR Green PCR Master Mix (Applied Biosystems). Primer Express software (Applied Biosystems) was used to design appropriate primers pairs for target genes [Nanog homeobox (Nanog), octamer-binding transcription factor (OCT) 3/4, IR, IGF-I, IGF-II, interferon-induced transmembrane protein (IFITM)1, and IFITM3] as well as for reference gene (glyceraldehyde-3-phosphate dehydrogenase) (Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). Relative quantitative determination of target gene levels was performed by comparing the comparative threshold cycle method (20).

Western blotting and immunoprecipitation

Cell lysates were prepared and processed as previously described (19). Membranes were incubated overnight with the following primary antibodies: anti-IGF-I-Rβ, anti-IRβ, anti-src homology 2 domain containing transforming protein (Santa Cruz Biotechnology, Inc.); antiphospho-Akt (Ser473), anti-AKT (v-akt murine thymoma viral oncogene homolog 1), antiphospho-glycogen synthetase kinase3β (Ser9), anti-glycogen synthetase kinase3β, and anti-ERK (Cell Signaling Technology, Beverly, MA); antiphospho-ERK (Tyr202/Tyr204) (Covance, Princeton, NJ); anti-insulin receptor substrate-1 (Upstate Biotechnology, Temecula, CA); anti-IFITM1 (Santa Cruz Biotechnology, Inc.); and antirabbit or antimouse antibodies conjugated to horseradish peroxidase (GE Healthcare, Piscataway, NJ) were used as secondary antibodies. For immunoprecipitation, lysates were immunoprecipitated as previously described (21).

The phosphorylation status of IRβ was measured with the anti-p-IGF-IRβ(Y1131)/p-IRβ(Y1146) (Cell Signaling Technology) in IRβ immunoprecipitation.

Neural differentiation

EWS cells were seeded at low density (25,000 cells/dish 60 mm) in standard medium; 96 h later, immunofluorescence analysis was performed for H neurofilament and β-III tubulin (Sigma, St. Louis, MO), as previously described (22).

IGF-IR internalization

Cells were plated onto 10 μg/ml fibronectin-coated glasses coverslips. After 1 d, cells were starved in binding buffer [IMDM 2% FBS, 20 mM HEPES (pH 7.4), and 0.1% BSA] with 2% FBS for 1 h; 200 μM chloroquine or 20 mM NH_4Cl was added when specified. Afterwards, cells were incubated at 37 C in the presence of AVE1642 HAb (100 ng/ml) or NVP-AEW541 (5 μg/ml) for 1–6 h and then fixed in 4% paraformaldehyde. Cells were permeabilized with 0.1% saponin. IGF-IR was detected by using HAb AVE1642 and antihuman Alexa Fluor 488-conjugated as secondary antibody. To stain lysosomes, lysosomal-associated membrane protein 1 (LAMP-1) monoclonal antibody (BD Pharmingen, San Diego, CA) was used as primary antibody, followed by a Cy3 antimouse secondary antibody (goat antimouse; Molecular Probes, Eugene, OR). Nuclei were 4′,6-diamidino-2-phenylindole stained. Analyses were made by confocal microscope.

Gene expression profiling and bioinformatics analysis

Cells were profiled by using HG-U133 Plus 2.0 array according to manufacturer's instruction (Affymetrix, Santa Clara, CA). Two/three independent experiments were performed for each cell line. Data were normalized and summarized by *rma* algorithm, filtered for low expressed genes, and analyzed with supervised techniques by *t* test statistics ($P \leq 0.05$) with Benjamini and Hochberg (23) correction to reduce false discovery rate (FDR) and/or by firstly considering fold changes (FC) of at least 2.0 between the mean of each compared group. Microarray data are available at GEO with accession no. GSE34027 (24). Hierarchical clustering was performed on differentially expressed genes using Pearson's correlation in GeneSpring 11.02 software. KEGG (25) pathways and Gene Ontology (26) were analyzed by MetaCore from GeneGO, Inc. (St. Joseph, MI) and by FatiGO (http://babelomics.bioinfo.cipf.es/) (27) softwares. Functional analysis was also performed on normalized data after exclusion of low-expressed genes with GSEA (www.broadinstitute.org/gsea/) (28).

Statistical analysis

Differences among means were analyzed using a two-sided Student's *t* test; gene variations were considered significant when $P \leq 0.05$.

Results

Cells resistant to anti-IGF-IR agents maintain the malignant features of parental cells

Starting from the sensitive TC-71 EWS cell line, we obtained a cell variant (named TC/CP) stably resistant to 100 μg/ml HAb Figitumumab (CP-751,871) (Supplemental Fig. 1). TC/CP cells were analyzed in comparison with parental cell line as well as TC-71 cell variants resistant to HAb AVE1642 and NVP-AEW541, which were previously characterized (11). All the three resistant variants maintained the proliferative and malignant properties of parental cell line, as shown by similar *in vitro* growth

capabilities (Fig. 1A), as well as expression of Oct3/4 and Nanog, two markers of cell stemness (Fig. 1B). Considering drug sensitivity, all the three resistant cell variants showed cross-resistance to the anti-IGF-IR agents (Fig. 1C) and similar levels of sensitivity toward vincristine and doxorubicin (Fig. 1D), two leader drugs in the treatment of EWS tumors.

Functional pathways associated with resistance to anti-IGF-IR agents

Gene expression profile of TC/AVE, TC/AEW, and TC/CP cells was performed using Affymetrix GeneChip and compared with that of parental cell lines. Genetic data were analyzed as summarized in Supplemental Fig. 2 and specified in *Materials and Methods*. Functional en-

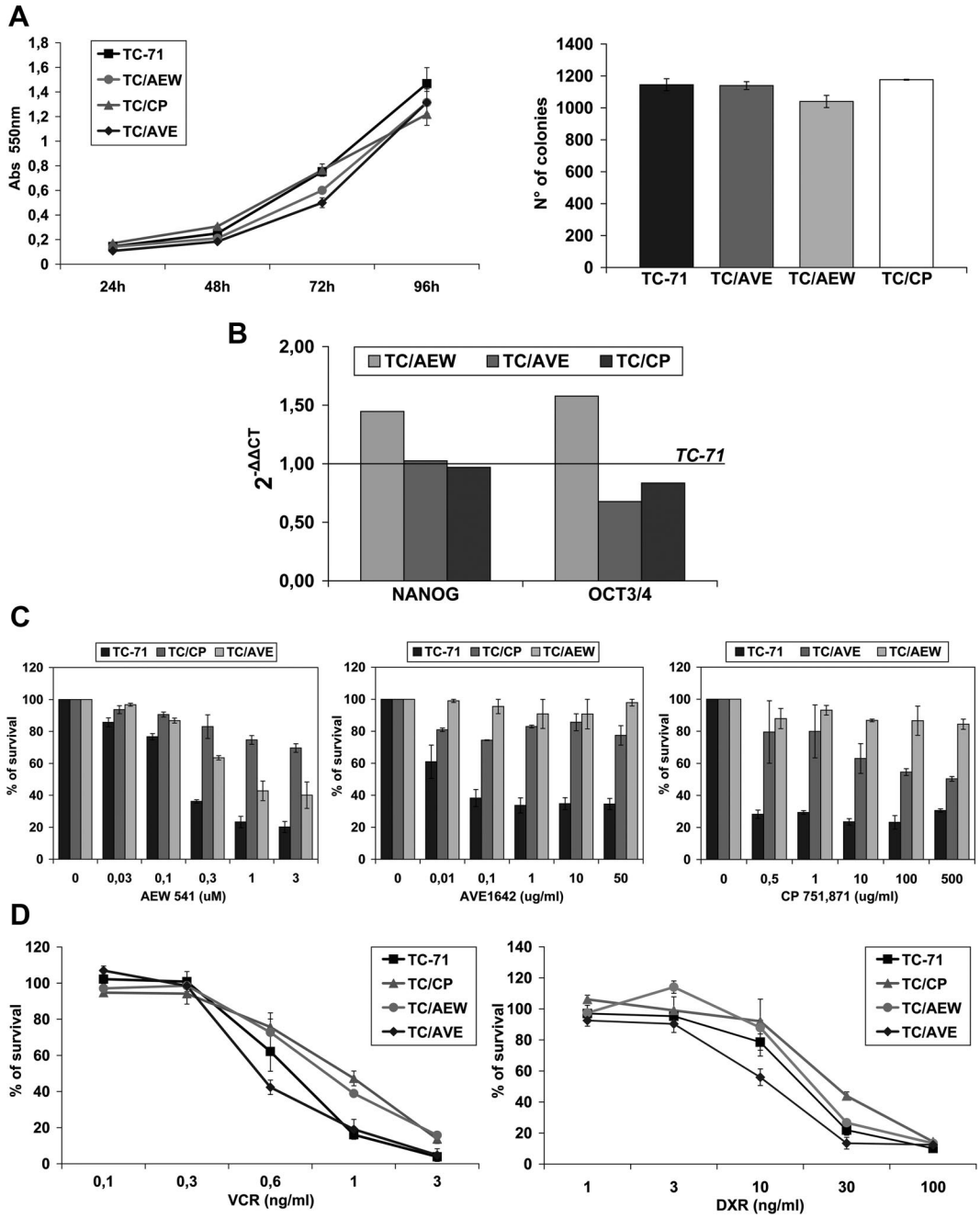

FIG. 1. Biological features of EWS cells resistant to different agents anti-IGF-IR. A, *In vitro* growth of resistant variants compared with parental cell lines. *Left*, Monolayer cell growth was assessed by MTT assay. *Right*, Anchorage-independent growth. Number of colonies in triplicate plates was determined after 7 d of growth. Data represent mean values of three independent experiments, and *bars* represent SE. B, Relative mRNA expression level of OCT 3/4 and Nanog, two markers of cell renewal and pluripotency, in resistant cell lines. TC-71 parental cells were used as calibrator ($2^{-DDCT} = 1$). C, Cross-resistance to different anti-IGF-IR agents: sensitivity was assessed by MTT assay after 72 h of exposure to either AVE1642 or CP-751,871 or NVP-AEW541. *Columns* represent mean values of three independent experiments, and *bars* represent SE. D, Efficacy of VCR or DXR in TC/CP, TC/AEW, and TC/AVE. Cell growth was assessed by MTT assay after 72 h of exposure to VCR or DXR and shown as a percentage of survival. Data represent mean values of three independent experiments and bars represent SE.

richment analysis by GSEA of the three resistant cell lines compared with sensitive parental cells identified a significant ($P < 0.05$ and FDR < 0.25) modulation of 33 common pathways (Table 1). We investigated in detail the most modulated pathways: MAPK kinase pathway ($P = 6.54E^{-3}$), insulin signaling ($P = 1.29E^{-2}$), and endocytosis ($P < 1.00E^{-4}$). Concerning insulin pathway, we have previously demonstrated a switch from a canonical IGF-I/IGF-IR-mediated pathway to the alternative IGF-II/IR-A signaling in cells resistant to HAb AVE1642 and NVP-AEW541 (11). Here, we confirm that also the TC/CP-resistant cells show up-regulation at transcriptional and protein level of IR-A (Fig. 2) as well as up-regulation of IGF-II, without significant changes in IGF-I and IGF binding protein-3 expression (Supplemental Fig. 3). In parallel, cells show down-regulation of IGF-IR (Fig. 2), a feature that may be due to alterations in endocytosis/degradation of the receptor or may simply reflect the mechanisms of action of anti-IGF-IR drugs. Either AVE1642 or Figitumumab HAb was reported to induce

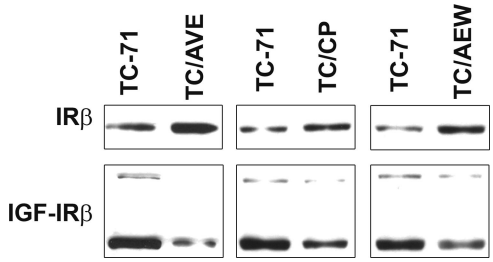

FIG. 2. IGF-IR/IR modulation in resistant variants compared with parental cells. Protein expression levels of receptors in resistant variants. Blots are representative of two independent experiments.

internalization and degradation of IGF-IR (29, 30), whereas no information is available for NVP-AEW541. We followed intracellular IGF-IR trafficking upon HAb AVE1642 or NVP-AEW541 treatments by immunofluorescence. In TC-71-untreated cells, IGF-IR mainly localizes at cell surface (Fig. 3A). Internalization route was tracked by colocalization of IGF-IR with the known protein marker LAMP-1 that stains lysosomes (Fig. 3A). After 1 h of treatment with AVE1642 HAb, IGF-IR was

TABLE 1. Pathways significantly modulated ($P < 0.05$ and FDR < 0.25) in EWS-resistant *vs.* EWS-sensitive cells according to GSEA analysis with Kegg database

NAME	Size	ES	*P* value	FDR
MAPK_SIGNALING_PATHWAY	134	−0.14	0.00654	0.04
INSULIN_SIGNALING_PATHWAY	78	−0.168	0.0129	0.06
ENDOCYTOSIS	91	−0.185	<0.0001	0.03
VEGF_SIGNALING_PATHWAY	46	−0.193	0.0383	0.14
NATURAL_KILLER_CELL_MEDIATED_CYTOTOXICITY	58	−0.203	0.0108	0.06
RENAL_CELL_CARCINOMA	41	−0.206	0.0301	0.15
CHEMOKINE_SIGNALING_PATHWAY	70	−0.214	<0.0001	0.04
GNRH_SIGNALING_PATHWAY	44	−0.214	0.0336	0.11
TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY	43	−0.215	0.0262	0.12
ERBB_SIGNALING_PATHWAY	48	−0.219	0.011	0.08
FC_GAMMA_R_MEDIATED_PHAGOCYTOSIS	45	−0.233	0.00518	0.06
PANCREATIC_CANCER	47	−0.244	0.00266	0.03
GLYCEROPHOSPHOLIPID_METABOLISM	28	−0.251	0.0362	0.15
ACUTE_MYELOID_LEUKEMIA	36	−0.252	0.00535	0.06
ANTIGEN_PROCESSING_AND_PRESENTATION	29	−0.256	0.0234	0.14
CELL_ADHESION_MOLECULES_CAMS	46	−0.257	0.0027	0.03
PATHOGENIC_ESCHERICHIA_COLI_INFECTION	28	−0.27	0.0227	0.12
T_CELL_RECEPTOR_SIGNALING_PATHWAY	59	−0.283	<0.0001	<0.01
FC_EPSILON_RI_SIGNALING_PATHWAY	38	−0.286	<0.0001	0.03
B_CELL_RECEPTOR_SIGNALING_PATHWAY	42	−0.3	<0.0001	0.01
ENDOMETRIAL_CANCER	32	−0.309	0.00494	0.03
COLORECTAL_CANCER	40	−0.313	<0.0001	0.02
N_GLYCAN_BIOSYNTHESIS	21	−0.342	0.00454	0.05
TYPE_II_DIABETES_MELLITUS	20	−0.35	0.00948	0.05
DORSO_VENTRAL_AXIS_FORMATION	14	−0.368	0.032	0.12
TYPE_I_DIABETES_MELLITUS	12	−0.399	0.0256	0.13
STEROID_BIOSYNTHESIS	16	−0.403	0.0112	0.04
BIOSYNTHESIS_OF_UNSATURATED_FATTY_ACIDS	14	−0.464	<0.0001	0.03
NITROGEN_METABOLISM	7	−0.499	0.0319	0.12
GLYCOSAMINOGLYCAN_BIOSYNTHESIS_KERATAN_SULFATE	6	−0.512	0.0413	0.18
GRAFT_VERSUS_HOST_DISEASE	10	−0.516	0.00462	0.04
AUTOIMMUNE_THYROID_DISEASE	11	−0.524	0.00444	0.03
ALLOGRAFT_REJECTION	10	−0.608	<0.0001	0.01

Pathways are ordered on the basis of enrichment score (ES) (lower ES indicates higher significance). Pathways with less than 5 or more than 200 genes were excluded because considered meaningless or nonspecific, respectively.

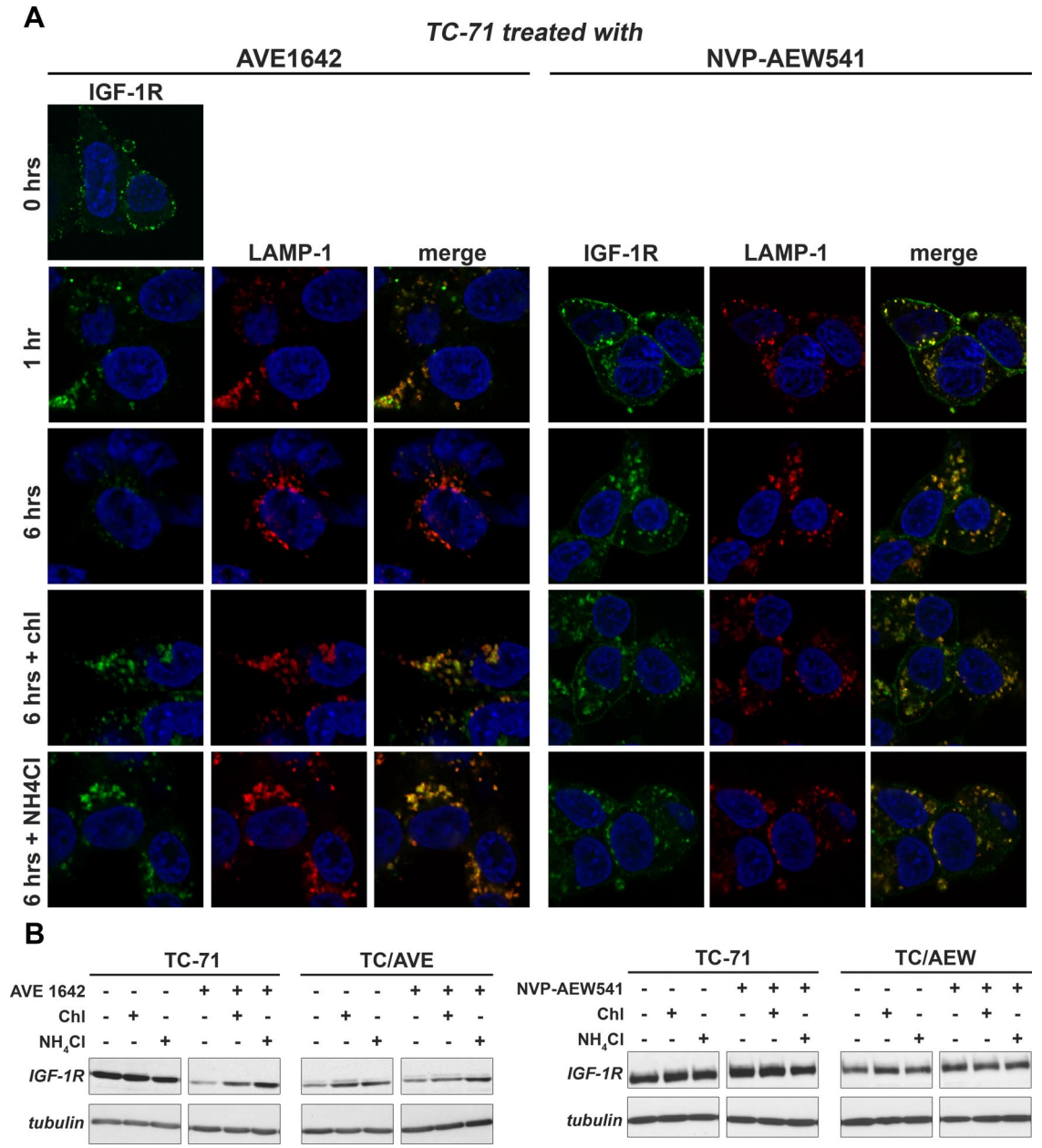

FIG. 3. Different IGF-IR route in resistant cells compared with parental cells. A, Fluorescence evaluation of IGF-IR internalization/degradation in TC-71 cells, upon AVE1642 or NVP-AEW541 treatment, as described in *Materials and Methods*. LAMP-1 staining was used as lysosome marker; Chl and NH_4Cl were used as lysosome function inhibitors. *Scale bars*, 50 μm. B, Modulation of IGF-IR protein levels in TC-71, TC/AVE, and TC/AEW cells upon 6 h of treatment with AVE1642 or NVP-AEW541 in combination or not with the lysosomal inhibitors (Chl and NH_4Cl). Tubulin was used as loading control. Blots are representative of two independent experiments.

completely internalized and contained in perinuclear round-shaped subcellular compartments. It colocalized largely with LAMP-1, suggesting that it has been routed to lysosomes. After a 6-h treatment, IGF-IR levels were significantly reduced due to receptor degradation, as observed also by western blotting analysis (Fig. 3B). To confirm that IGF-IR degradation was mainly dependent on the lysosomal pathway, we used two inhibitors of lysosomial-dependent degradation, chloroquine and NH_4Cl (31). Receptor degradation was indeed prevented, as indicated by the accumulation of IGF-IR in large perinuclear compartments that colocalized with LAMP-1 (Fig. 3A) as well as by the restoring of IGF-IR protein level (Fig. 3B). Similar effects were observed in TC/AVE cells. Although weaker at baseline conditions, IGF-IR expression became more marked after combined treatment of resistant cells with AVE1642 HAb and lysosomal inhibitors (Fig. 3B). This indicates that AVE1642 HAb similarly induces internalization and degradation of IGF-IR in sensitive and resistant cells. In contrast, comparative analysis

with NVP-AEW541 indicates that treatments with the TKI lead to receptor internalization but not to IGF-IR degradation. After a 6-h treatment, receptor localizes in discrete foci, with a good LAMP-1 colocalization (Fig. 3A), but no reduction of IGF-IR levels is observed (Fig. 3, A and B). Cotreatment with chloroquine or NH_4Cl does not modify the intracellular localization of the receptor (Fig. 3A) nor its protein expression levels (Fig. 3B). After a 20-h treatment with the TKI, receptor relocalizes at cell membrane (Supplemental Fig. 4), possibly through recycling, in agreement with the idea that NVP-AEW541 induces internalization but not degradation of IGF-IR. Similar effects were observed in TC/AEW-resistant cells. Exposure to lysosome inhibitors did not modify the expression of IGF-IR (Fig. 3B), indicating also in this case no substantial alteration in the IGF-IR internalization/degradation processes between resistant and sensitive cells. Exposure to HAb AVE1642 or to TKI NVP-AEW541 induces inhibition of MAPK and AKT pathway in sensitive but not in resistant cells, as expected (data not shown). Thus, although in TC/AVE cells, the lower expression of IGF-IR compared with parental cell line may reflect the constant and higher activation of the endocytosis route by the antibody, the IGF-IR down-regulation in TC/AEW cells is attributable to other mechanisms, likely involving decreased IGF-IR transcription as previously shown (11). Concerning IR signaling, we did not observe remarkable differences among the three resistant cell variant with respect to IR-A phosphorylation status (Fig. 4A). Regarding MAPK pathway, functional analysis highlighted that all the genes participating to MAPK signaling are commonly up-regulated in resistant cells (Supplemental Fig. 5), whereas genes of the AKT pathway have a more variable behavior. Indeed, a constitutive hyperphosphorylation of ERK1/2 was observed in all the three resistant cell variants as compared with the parental cells (Fig. 4B), whereas AKT pathway activation seems more variable in resistant cells, being reduced in TC/CP and TC/AEW but slightly increased in TC/AVE (Fig. 4B). Taken together, these data indicate that all three resistant cell lines develop an unbalanced activation of IR-A-mediated intracellular pathways that favors the MAPK pathway over the AKT pathway in sustaining malignant features and resistance to anti-IGF-IR agents.

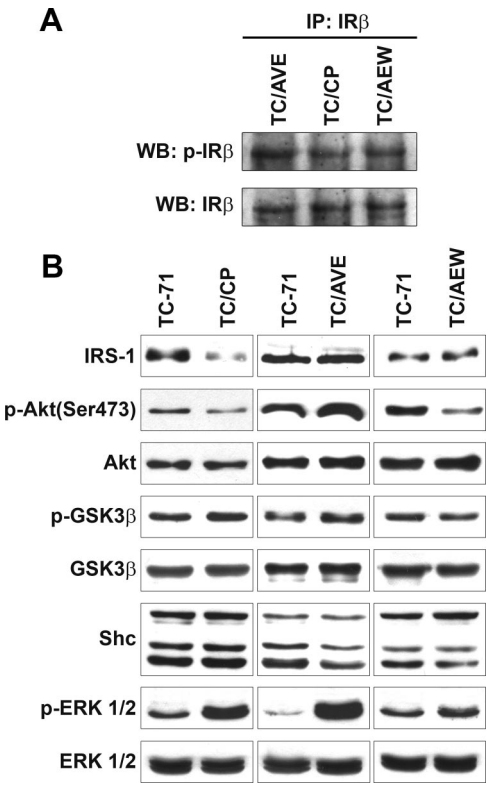

FIG. 4. IGF system modulation in resistant variants compared with resistant cells. A, Phosphorylation level of IR-A between resistant cells. Total protein lysates (500 μg) were immunoprecipitated (IP) with IRβ polyclonal antibody and then probed (Western blotting) by anti-p-IGF-IRβ(Y1131)/p-IRβ(Y1146). B, Activation status of IGF-IR/IR signaling mediators in parental and resistant cells. Total proteins were used as controls. Blots are representative of two independent experiments. IRS, Insulin receptor substrate; Shc, src homology 2 domain containing transforming protein; GSK, glycogen synthase kinase.

Common gene signatures associated with resistance to anti-IGF-IR agents

Supervised analysis by *t* test corrected for FDR was used for the detection of differentially expressed genes in cells sensitive or resistant to anti-IGF-IR drugs. It pointed out a genetic signature of 191 probes (91 up-regulated and 100 down-regulated), corresponding to 160 unique well-characterized genes (for complete list of probes, see Supplemental Table 2). Clustering algorithm analysis performed with this signature correctly distinguishes resistant cells (Fig. 5A). Top regulated genes are shown in Fig. 5A, indicating alterations in genes functionally associated with cholesterol biosynthesis and lipid metabolism, inflammation (up-regulated genes), or with cytoskeleton remodeling and extracellular matrix interaction (down-regulated genes). Involvement of these functions was pointed out also by annotation analysis performed by GeneGO Software (data not shown).

To further highlight common mechanisms that may cause and/or sustain resistance to different anti-IGF-IR therapies, we also analyzed each of the three cell variants separately in comparison with the parental cell line. FC evaluation (>2 FC) and *t* test analysis ($P < 0.05$) ended up with the definition of selective gene expression profiles associated with resistance to HAb AVE1642 (signature of 562 probes), HAb CP-751,871 (signature of 385 probes), and NVP-AEW541 (signature of 166 probes) (detailed lists of genes reported in Supplemental Tables 3–5). Over-

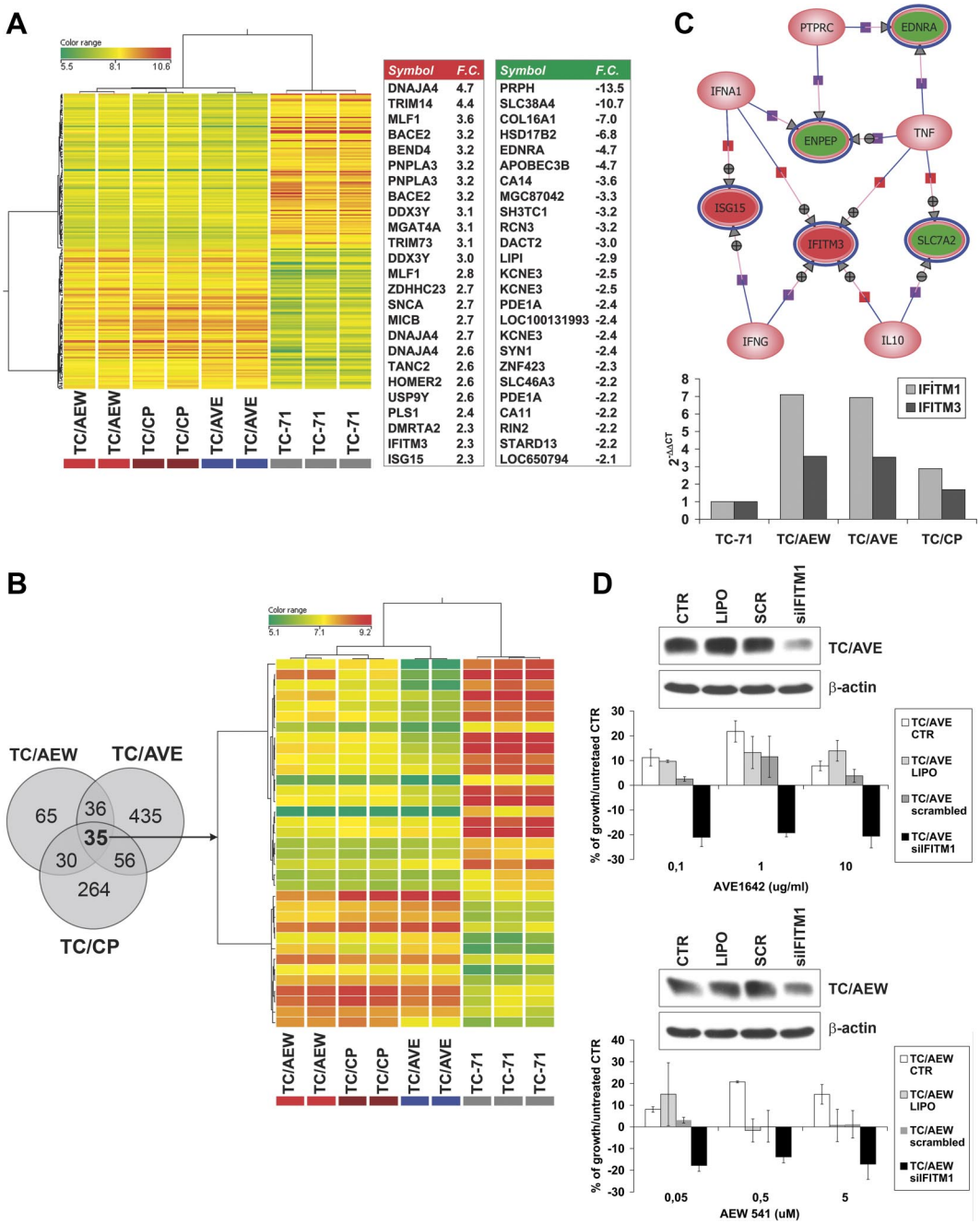

FIG. 5. Common gene signatures associated with resistance to anti-IGF-IR agents. A, Cluster analysis performed with the 191 probes differentially expressed between resistant variants *vs.* parental TC-71 cell line (Supplemental Table 2). The most up-regulated and down-regulated genes with their relative FC, fold changes, are shown. B, Venn diagram shows common genes to the three distinctive signatures that emerged from separate microarray analysis of each resistant cell line *vs.* parental TC-71 (Supplemental Tables 3–5); 35 probes were in common and cluster analysis distinguished resistant cell lines. C, Network analysis on the 35 probes pointed out a common network based on interferon-induced transmembrane molecule IFITM3. Its up-regulation, together with that of IFITM1, was confirmed in resistant variants by quantitative RT-PCR. D, Reversion of resistance to anti-IGF-IR therapies by IFITM1 knockdown. Silencing of IFITM1 in TC/AVE and TC/AEW cell lines was achieved after 24 h of transfection of siIFITM1 (50 nM) or scrambled control siRNA (50 nM); β-actin was used as loading control. Transfected cells were treated as described in *Materials and Methods*. Cell survival was shown as a percentage of growth respect to untreated control. Data represent mean values of two independent experiments, and bars represent SE.

lapping of these three signatures indicated a core group of 35 probes that appeared to be in common to the three resistances (Fig. 5B). This restricted signature was still able to clusterize the three resistant cell variants in comparison with parental cell line (Fig. 5B). Among these 35 probes, 27 were also included in the common 191-probe signature and are reported in Table 2. Among most modulated genes, IFITM3 and ubiquitin-like modifier [ISG

TABLE 2. Thirty-five probes with significant differential expression in all three cell lines

Probe	Symbol	Description	FC TC/AVE *vs.* TC-71	FC TC/AEW *vs.* TC-71	FC TC/CP *vs.* TC-71
204783_at[a]	MLF1	Myeloid leukemia factor 1	4.1	4.4	2.5
217867_x_at[a]	BACE2	β-Site APP-cleaving enzyme 2	3.7	2.7	3.2
1554250_s_at[a]	TRIM73	Tripartite motif-containing 73	3.7	2.7	2.8
217080_s_at[a]	HOMER2	Homer homolog 2 (*Drosophila*)	3.2	2.2	2.5
230896_at[a]	BEND4	BEN domain-containing 4	3.1	3.3	3.1
233030_at[a]	PNPLA3	Adiponutrin	2.8	3.2	3.7
220675_s_at[a]	PNPLA3	Adiponutrin	2.8	3.1	3.8
1554334_a_at[a]	DNAJA4	DnaJ (Hsp40) homolog, subfamily A, member 4	2.8	2.3	3
226912_at[a]	ZDHHC23	Zinc finger, DHHC-type-containing 23	2.6	2.8	2.8
212203_x_at[a]	IFITM3	Interferon-induced transmembrane protein 3 (1-8U)	2.3	2.6	2.1
235099_at	CMTM8	CKLF-like MARVEL transmembrane domain-containing 8	2.1	3.8	4.2
205483_s_at[a]	ISG15	ISG15 ubiquitin-like modifier	2.1	2.5	2.3
231213_at[a]	PDE1A	Phosphodiesterase 1A, calmodulin dependent	−2.1	−2.2	−2.3
222923_s_at[a]	KCNE3	Potassium voltage-gated channel, Isk-related family, member 3	−2.2	−2.4	−2.8
222922_at[a]	KCNE3	Potassium voltage-gated channel, Isk-related family, member 3	−2.3	−2.4	−2.5
236234_at[a]	PDE1A	Phosphodiesterase 1A, calmodulin dependent	−2.6	−2.4	−2.4
202712_s_at[a]	CKMT1A	Creatine kinase, mitochondrial 1A	−3.1	−2.3	−2.4
1569648_at[a]	DACT2	Dapper, antagonist of β-catenin, homolog 2 (*Xenopus laevis*)	−3.4	−2.6	−3
217553_at[a]	MGC87042	STEAP family protein MGC87042	−3.4	−3.2	−3.2
61734_at[a]	RCN3	Reticulocalbin 3, EF-hand calcium-binding domain	−4.1	−3	−2.6
219837_s_at	CYTL1	Cytokine-like 1	−4.5	−2.1	−2.9
219464_at[a]	CA14	Carbonic anhydrase XIV	−4.9	−3.2	−3
202465_at	PCOLCE	Procollagen C-endopeptidase enhancer	−5.1	−2.3	−2.6
204818_at[a]	HSD17B2	Hydroxysteroid (17-β) dehydrogenase 2	−6.3	−7.9	−6.2
204464_s_at[a]	EDNRA	Endothelin receptor type A	−7.4	−3.1	−4.6
206632_s_at[a]	APOBEC3B	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3B	−7.6	−3.8	−3.5
204345_at[a]	COL16A1	Collagen, type XVI, α 1	−7.8	−6.5	−6.7
225516_at	SLC7A2	Solute carrier family 7, member 2	−8.2	−2.1	−2.6
213479_at	NPTX2	Neuronal pentraxin II	−15.4	−2.4	−4.9
220786_s_at[a]	SLC38A4	Solute carrier family 38, member 4	−16.2	−7.8	−9.8
213847_at[a]	PRPH	Peripherin	−16.8	−11.7	−12.4
204844_at	ENPEP	Glutamyl aminopeptidase (aminopeptidase A)	−17.5	−2.5	−2.1

All probes have same modulation in all three experiments. Three probes (1554220_a_at, 237226_at, and 228045_at) match no coding regions and were excluded from the table; the first probe was up-regulated in resistance cells, whereas the remaining two probes were down-regulated.

[a] Probes that were also identified in previous signature of 191 probes.

(interferon-stimulated gene) 15] were up-regulated, whereas glutamyl aminopeptidase A (ENPEP), endothelin receptor type A (EDNRA), and solute carrier family (SLC)7 member 2 were strongly down-regulated. Comparative network analysis highlighted a common network based on IFITM gene family (Fig. 5C). Increased expression of the IFITM family members IFITM1 and IFITM3 was validated by RT-PCR in resistant cell lines (Fig. 5C), thus identifying another common circuit related to IGF-IR resistance. Silencing of IFITM1 by short hairpin RNA technology supported its functional role in mediating IGF-IR resistance. TC/AVE or TC/AEW were pretreated with IFITM1 siRNA for 24 h and then exposed to AVE1642 or to NVP-AEW541 for 48 h. As shown, in Fig. 5D, when cells are deprived of IFITM1 their sensitivity to anti-IGF-IR agents is partially recovered.

Differential and specific gene profiles associated with resistance to anti-IGF-IR drugs

In addition to identifying shared resistance mechanisms, we were also interested in pointing out possible mechanisms/pathways of resistance unique to the two types of IGF-IR inhibitors (HAb *vs.* TKI). Functional annotation analysis of the gene expression profiles specifically associated with resistance to the three agents (detailed lists of genes reported in Supplemental Tables 3–5) indicated that diverse pathways/processes were modulated in cells resistant to anti-IGF-IR HAb as compared with cells resistant to the TKI NVP-AEW541. In particular, either GeneGO (Fig. 6A) or FatiGO softwares (Fig. 6B) pointed out that resistance to HAb involves mainly genes regulating neural differentiation and angiogenesis, whereas resistance to NVP-AEW541 is mainly associated

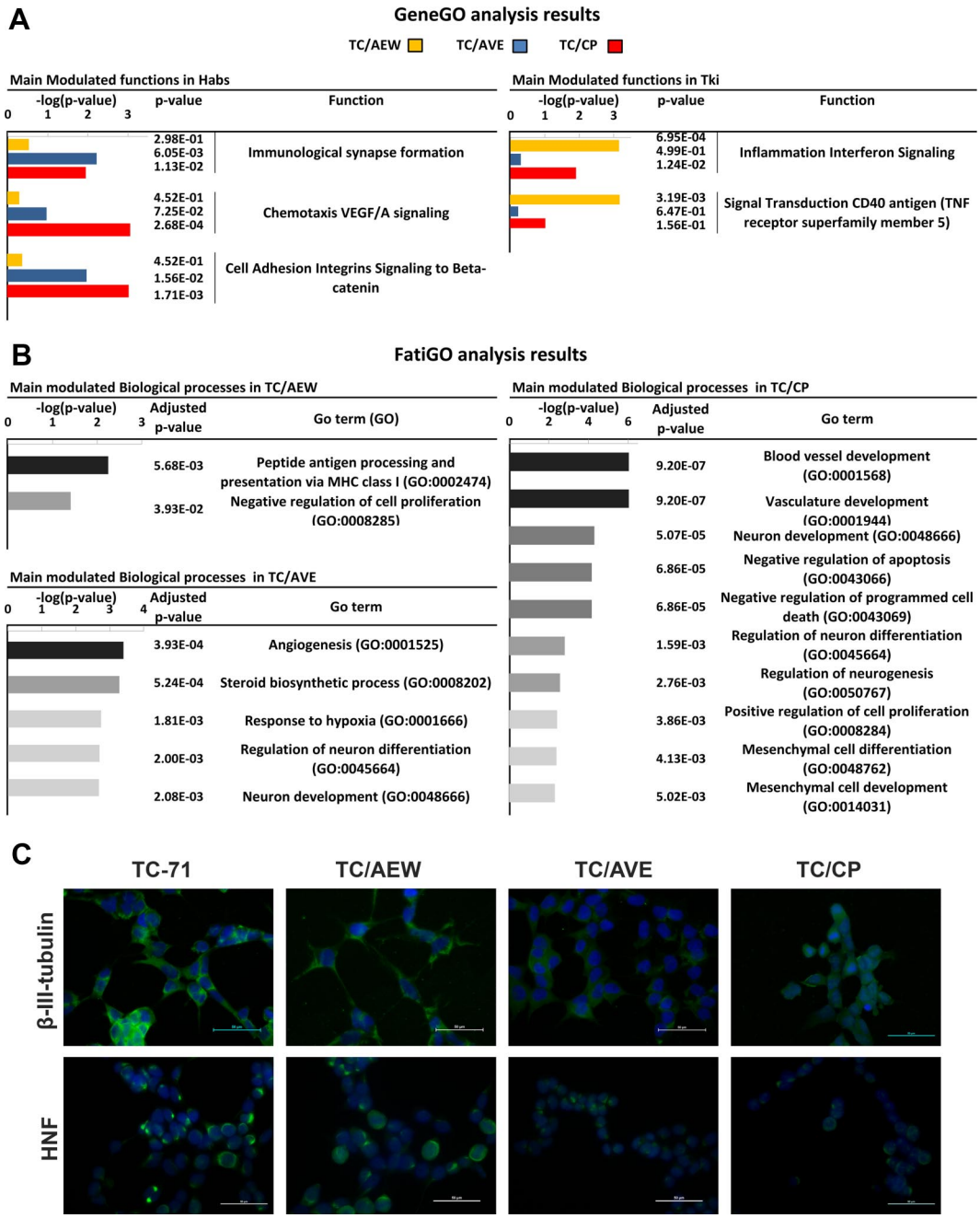

FIG. 6. Distinctive processes associated with resistance to different anti-IGF-IR drugs (HAb *vs.* TKI). Functional annotation analysis of the three distinctive signatures that discriminate each resistant cell line from the parental TC-71 (Supplemental Tables 3–5) by GeneGO (A) or FatiGO (B) identified the mostly significantly modulated functions. Cells resistant to HAb mainly showed modulation of genes related to neuronal differentiation, angiogenesis, and cell adhesion, whereas cell resistant to TKI are more characterized by modulation of genes associated with immunoresponse and antigen presentation. C, Specific inhibition of neural differentiation in cells resistant to anti-IGF-IR HAb. Neurofilament (H-NF) and β-III tubulin expression was evaluated in cells maintained in standard culture conditions. Representative photomicrographs are shown for sensitive and resistant variants. *Scale bars*, 50 μm.

with alterations in genes concerning inflammation and antigen presentation as well as in stress responses. Consistently, we observed inhibition of neural differentiation in TC/CP and TC/AVE cells compared with TC/AEW or TC-71 cells, according to the expression of β-III tubulin and 200-K neurofilament, two specific markers of neural differentiation (Fig. 6C).

Discussion

Several experimental and clinical evidences now clearly indicate that, in addition to the IGF-IR, also the IR is involved in cancer (32). The functional specificity of insulin/IR signaling is in fact changed in cancer cells because of the predominant production of isoform A (IR-A) that

has increased binding affinity for IGF, enhanced hybrid receptor formation between IR and IGF-IR, and autocrine and/or paracrine IGF production. However, due to concerns on toxicity and possible development of hyperglycemia because of the high homology between IGF-IR and IR, IR has not been considered as a therapeutic target in cancer, and the initial choice has been the development of highly selective anti-IGF-IR agents. Single-agent activity has been reported particularly in EWS, but only a minority of patients really benefit from this targeted therapies, thus prompting the scientific community to identify the mechanisms of native and acquired resistance to these agents to individualize their use in clinics. Because tumor cells exhibit a high degree of signaling plasticity, which can contribute to adaptive survival in the presence of a receptor inhibitor, we compared gene expression profiles of cells made resistant to three different anti-IGF-IR drugs (the HAb AVE1642, Figitumumab, or the TKI NVP-AEW571) to highlight common and distinctive mechanisms of resistance. We identified two molecular signatures, one of 191 probes and one more restricted of 35 probes that perfectly distinguish sensitive from resistant cells. The annotation analysis indicated some common altered pathways, mainly related to insulin signaling, MAPK pathway, endocytosis, and dysregulation of the interferon system. Because one interesting common feature about the antibodies directed against IGF-IR is their ability to bind and down-regulate IGF-IR level through receptor-mediated endocytosis (30, 33), we explored possible alterations in the IGF-IR endocytosis processes. Endocytosis is tightly related with signaling transduction, modulating and/or sustaining it (34). In EWS cells, IGF-IR is internalized upon ligand binding, and this process, which is dependent on either clathrin-mediated or raft/caveolar endocystosis, is important for signaling (35). Here, we show that resistant cells seem to maintain intact the IGF-IR internalization/degradation route of sensitive cells. However, down-regulation of IGF-IR is a common feature of cells resistant to anti-IGF-IR agents. Most likely, in cells resistant to anti-IGF-IR HAb, this may rely on higher functioning of the lysosomial degradative pathway, which enhances the overall down-regulation of the receptor, whereas this process is only partially observed in cells resistant to the TKI NVP-AEW571, which retain only the ability to internalize the receptor but not to route it to degradation. In these cells, the down-regulation of IGF-IR seems to be due to limited transcription of IGF-IR (11). Whether or not, in analogy with the well-studied EGF receptorial model (36), this may depend on the existence of a specific ubiquitination code that, in turns, determines the receptor fate upon different stimulation, it will be the subject of future biological studies. In any case, from a practical point of view, the loss of proliferative stimulus, normally offered in EWS cells by IGF-IR constantly activated by IGF-I autocrine production, resulted compensated in resistant cells by the switch to IGF-II/IR-A dependency. In this article, we extended our previous observations (11) and confirmed that whenever EWS cells are exposed to a truly specific anti-IGF-IR agent, either HAb or TKI, they may adapt by inducing compensatory activation of an IR-A-dependent pathway. Transcriptional up-regulation of IR-A and IGF-II is thus a common mechanism of resistance to anti-IGF-IR agents in EWS. In addition, we observed common up-regulation of all the major mediators of MAPK/ERK pathway in the three resistant variants, in keeping with the maintenance of growth abilities and stemness features of parental cells. Interestingly, Giudice *et al.* (37) have recently found a higher rate of endocytosis of IR-A, compared with IR-B, the isoform associated to metabolic functions of insulin, and have correlated these differences with preferential ERK1/2 response to insulin in cells expressing IR-A in contrast to preferential AKT response to insulin in cells expressing IR-B. The higher IGF-IR endocytosis/degradation that we observed in resistant cells may thus be responsible for their preferential activation of ERK1/2, because previous findings have shown that long-term cell exposure to AVE1642 resulted in endocytosis of the IR present in lipid rafts along with IGF-IR (33). In addition, using R-/IR-A cells, which represent a suitable model to study the effect of IGF on IR-A, Sacco *et al.* (38) found that IGF-II elicits a peculiar signaling pattern characterized by high ERK1/2 response in contrast to lower AKT activation compared with insulin. The compensatory role of MAPK signaling after blockage of IGF-IR was also recently highlighted when EWS cells were exposed to the anti-IGF-IR R1507 HAb (39), further supporting the use of MAPK inhibitors as adjuvant agents to overcome resistance.

Thanks to the exclusive expression of the isoform A of IR in EWS cells (11), together with the low/absent expression of IGF-IR in resistant variants, EWS may be considered a suitable model to study the differential effects of IGF-II compared with insulin. This model may also be exploited to clarify mechanisms by which IR-A overexpression and autocrine/paracrine production of ligands may contribute to the activation of the IGF system in cancer. This seems to be a crucial aspect either to define biomarkers for individualizing this targeted therapy or for a better understanding of the IGF system, which besides influencing cancer growth risk (40) greatly modulates cancer cell response to conventional and new drugs (41, 42). In fact, depending on receptor subtype expres-

sion and IGF-II *vs.* IGF-I local availability, a variety of conditions may be envisaged. For instance, if IR-A and IGF-IR are concomitantly overexpressed and IGF-I and IGF-II are present in similar molar concentrations, IGF-I will mostly signal through IGF-IR and hybrid receptors, and IGF-II through IR-A, due to their different affinities to cognate receptors. However, if local IGF concentration is in favor of one of the two ligands, interactions with receptors may change. Besides considering the ratio IR/IGF-IR (10–12), it is also mandatory to precisely evaluate expression of ligands. As we have previously shown (11), the two receptors are not equal in sustaining cell proliferation. In a Ewing's cell line producing only IGF-I, and in which the expression of IGF-IR and IR is similar, the silencing of IR-A has more limited effects than silencing of IGF-IR on proliferation. It is only when the cells started to produce IGF-II that the proliferative stimulus of IR-A was equal to that of IGF-IR. This means that differences between IR-A and IGF-IR effects may be due to a modulation of the amplitude of the signal created by the specific ligand-receptor interaction (43) and that quantitative analysis of receptor should go along with that of ligands to predict responses to therapy.

Several other papers have previously reported that expression of IGF-IR itself or other IGF system mediators correlate with response to IGF-IR blockade (13, 44–47). However, each of these papers supported one or the other mediator as predictive biomarker. Instead, our message is that, due to the complex cross talk between the different components of IGF system, all the players should be evaluated and monitored individually before planning treatments. As stated by Baserga in his review (48), customizing the targeting of IGF system seems to be a strict requirement for future clinical studies. Technology is now making this possible. For example, only patients with IR:IGF-IR and IGF-I:IGF-II ratios in favor of IGF-IR and IGF-I should be treated with anti-IGF-IR agents. For the others, that include tumors with aberrant expression of IGF-II, such as Wilms tumors, hepatocellular cancers, rhabdomyosarcoma, and osteosarcoma, dual inhibitors should be preferred.

Together with activation of IR-A/IGF-II signaling, we identified in modulation of some members of IFITM family another common mechanism of resistance to anti-IGF-IR agents. IFITM genes are still poorly studied molecules that serve different functions in different tumors, and whose exact role in tumorigenesis is complex (for a review, see Ref. 49). Although deeper studies are necessary, it is intriguing that these molecules have been found to be associated with resistance to several other anticancer drugs, such as interferon, radiotherapy, and drugs that target growing cells. We have also previously documented an increase in IFITM when EWS cells became resistant to Yondelis (50). Besides a role in the control of proliferation and cell adhesion, new functions have been recently identified that are related to cell differentiation and innate immunity. Interestingly, gene expression profiling data of our resistant cells indicated distinctive modulation in genes associated with antigen presentation and interactions with immune system for cells resistant to the TKI inhibitor NVP-AEW541, whereas modulations of genes associated with cytoskeleton, mesenchymal-epithelial transition, and neural differentiation appear to be more pronounced in cells resistant to HAb anti-IGF-IR. Whether and how these processes may be related to different expression and modulation of IFITM proteins, which serve different functions in different cellular contexts, will be the subject of future studies, but we still have to include IFITM proteins in the group of molecules that may have a crucial role in modulating drug resistance.

In conclusion, although the IGF system clearly remains an important therapeutic target, it is becoming clear that the complexity of this pathway requires analytical evaluation of its components to better select the patients and guide individualized treatment combination. Our data provide evidences on common as well as distinctive mechanisms of action and resistance to truly specific anti-IGF-IR agents and support the idea that using small molecules that cotarget IGF-IR and IR-A, such as TKI OSI-906 (12), or antibodies that target IGF-I and IGF-II, such as MEDI-573 (51), may provide new impetus for a more effective application of this targeted therapy.

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