Identification of 14-3-3β Gene as a Novel miR-152 Target Using a Proteome-based Approach*

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Background: miR-152 regulates HLA-G and HLA-C, which act inhibitory to NK and T cells, thereby altering the immunogenicity of tumors.

Results: Applying a proteome-based approach, novel miR-152 targets were identified, e.g. anti-apoptotic 14-3-3β overexpressed in certain tumors.

Conclusion: The known tumor-suppressive miR-152 regulates 14-3-3β, thereby enhancing the sensitivity of tumor cells for apoptosis.

Significance: miR-152 exerts a dual role by altering the immunogenicity and the tumorigenicity.

Recent studies demonstrated that miR-152 overexpression down-regulates the nonclassical human leukocyte antigen (HLA) class I molecule HLA-G in human tumors thereby contributing to their immune surveillance. Using two-dimensional gel electrophoresis followed by MALDI-TOF mass spectrometry, the protein expression profile of HLA-G+, miR-152low cells, and their miR-152-overexpressing (miRhigh) counterparts was compared leading to the identification of 24 differentially expressed proteins. These were categorized according to their function and localization demonstrating for most of them an important role in the initiation and progression of tumors. The novel miR-152 target 14-3-3 protein β/α/YWHAB (14-3-3β) is down-regulated upon miR-152 overexpression, although its overexpression was often found in tumors of distinct origin. The miR-152-mediated reduction of the 14-3-3β expression was accompanied by an up-regulation of BAX protein expression resulting in a pro-apoptotic phenotype. In contrast, the reconstitution of 14-3-3β expression in miR-152high cells increased the expression of the anti-apoptotic BCL2 gene, enhances the proliferative activity in the presence of the cytostatic drug paclitaxel, and causes resistance to apoptosis induced by this drug. By correlating clinical microarray data with the patients’ outcome, a link between 14-3-3β and HLA-G expression was found, which could be associated with poor prognosis and overall survival of patients with tumors. Because miR-152 controls both the expression of 14-3-3β and HLA-G, it exerts a dual role in tumor cells by both altering the immunogenicity and the tumorigenicity.

MicroRNAs (miRs)3 are small 22-nucleotide-long noncoding RNAs representing key regulators of the post-transcriptional gene regulation (1). So far, more than 2500 human miRs are listed at the mirbase on-line database (2). The sequence-specific binding to a target mRNA is determined by the “seed” region of the miRs (3) and predominantly occurs at the 3’-untranslated region (UTR) of the target mRNA.

Recently, members of the miR-148 family, miR-148A, miR-148B, and miR-152, have been demonstrated to regulate the immune modulatory nonclassical human leukocyte antigen (HLA) class I molecule, HLA-G (4). Under physiologic conditions, HLA-G is selectively expressed on fetal tissues, thereby regulating the feto-maternal immune tolerance, and in adults on immune-privileged organs. During the last few years, a role of HLA-G as immune modulatory molecule in several diseases such as autoimmune disorders, viral infections, and tumors has been described. HLA-G expression was often detected on solid and hematopoietic tumors, which could be associated with disease progression and poor patient survival (5–7). In addition to the regulation of HLA-G, the HLA-C antigen is also regulated by members of the miR-148 family (8). Because HLA-G and HLA-C are both ligands for inhibitory NK cell receptors (9, 10), the miR-148 family is an important regulator of an effective immune response against tumor cells and also against viral infections. The expression of miR-148 family members, including miR-152, is often down-regulated in tumors of distinct origin, including prostate, ovarian, endometrial, and colorectal cancers. This was associated with advanced tumor staging and grading as well as reduced overall survival of tumor patients. In contrast, high miR-152 expression levels were associated with increased apoptosis, decreased cell proliferation, invasion, and angiogenesis (11–14). Furthermore, plasma levels of miR-152 in tumor patients could be used as predictors of patient outcome (15). This is in line with the association of decreased miR-

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§ The abbreviations used are: miR, microRNA; CFSE, carboxyfluorescein succinimidyl ester; luc, luciferase; RCC, renal cell carcinoma; 2DE, two-dimensional gel electrophoresis; qPCR, quantitative PCR; ACN, acetonitrile.
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152 expression and chemotherapeutic resistance. Thus, miR-152 represents the tumor suppressor miR, which is often silenced by DNA hypermethylation in tumors (12). The following question is addressed. Which genes relevant for tumor cell fate and tumor progression are directly regulated by miR-152? miR-152 was overexpressed in the miR-152-low HLA-G+ choriocarcinoma cell line JEG-3 (4, 16). Because miRs impair protein synthesis from targeted mRNAs, 2DE-based proteomic approaches in combination with mass spectrometry were employed to identify novel miR-152 targets by comparative analyses of the protein expression patterns of miR-152-low/high tumor cells. One of these targets, 14-3-3β (14-3-3 protein β/α/YWHAB), was validated and functionally characterized. miR-152-mediated inhibition of 14-3-3β expression was accompanied by reduced growth capacity and enhanced apoptosis sensitivity in the presence of the chemotherapeutic drug paclitaxel, which could be reverted by restoration of 14-3-3β expression via down-regulation of the pro-apoptotic protein BAX and the up-regulation of the expression of the anti-apoptotic gene BCL2. In addition, 14-3-3β and HLA-G expression in selected tumor entities was linked to a reduced survival of patients.

MATERIALS AND METHODS

Cell Lines and Tissue Culture—The HLA-G negative human embryonal kidney cell line HEK293T and the HLA-G positive choriocarcinoma cell line JEG-3 were purchased from the American Type Culture Collection (ATCC® CRL-3216™ and ATCC® HTB-36™, Manassas, VA). The cell lines MZ1257RC, MZ1795RC, and MZ1851RC as well as buf1088, FM82, and WM1862 were established from patients with renal cell carcinoma (RCC) or metastatic melanoma, respectively, and have been described recently (17–20). With the exception of JEG-3 cells, which were maintained in RPMI 1640 medium (Invitrogen), all other cell lines were cultured in DMEM (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FCS) (PAA; Land), and 1% (v/v) penicillin/streptomycin (PAA).

Isolation of DNA, RNA, and miR—DNA and total cellular RNA were isolated using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) and the TRIzol reagent (Invitrogen) according to the manufacturers’ protocols, respectively. RNA was treated with DNase I (New England Biolabs) for 30 min at room temperature, inactivated with EDTA (5 mM final concentration), and then incubated at 75 °C for 10 min.

2DE, Protein Visualization, and Image Analysis—Frozen cell pellets (1 × 10^7 cells/sample; three biologic replicates) were harvested, washed three times in PBS (PAA), and stored at −80 °C. Proteins were extracted with lysis buffer in 7 M urea (AppliChem, Darmstadt, Germany), 2 M thiourea (Sigma), 0.2 M dimethylbeylammonium propane sulfonate (NDSB 201, Merck), 1% dithiothreitol (DTT; AppliChem, Darmstadt, Germany), 4% CHAPS (AppliChem), 0.5% Phosphalyte (Amersham Biosciences), and a trace of bromphenol blue (Serva Electrophoresis, Heidelberg, Germany). The lysate was sonicated using two cycles of five impulses (0.5 s/impulse) at 100% power (Bandelin UW 2070 sonicator, MS 73 needle; Bandelin, Berlin, Germany) and then cleared by centrifugation (18,000 × g, 90 min, 15 °C). Total protein concentration was determined as described previously (19). Samples (500 µg of protein in a volume of 350 µl of lysis buffer) were applied to IPG strips (pH 3–10 NL, 18 cm, GE Healthcare) by in-gel rehydration and covered with 450 µl of Immobiline DryStrip Cover Fluid (GE Healthcare). After 2 h of rehydration, isolectric focusing was carried out at 20 °C on an Ettan IPGphor 2 unit (GE Healthcare) at the following settings: 30 V for 10 h, 500 V for 1 h, 1000 V for 1 h, 5000 V for 1 h, and 8000 V up to a total of 45,000 V*h. The IPG strips were subjected to the strip equilibration procedure, which was performed by incubating the strips for 15 min in 12 ml of equilibration buffer (6 M urea, 2% SDS, 50 mM Tris-HCl (pH 8.8), 30% glycerol) supplemented with 1.5% DTT followed by 15 min in 12 ml of equilibration buffer supplemented with 4.8% iodoacetamide (all chemicals by AppliChem). SDS-PAGE separation was performed using a PROTEAN plus Dodeca Cell (Bio-Rad) with gels of 1.5 mm thickness and an acrylamide concentration of 13%. Strips were fixed on vertical SDS-polyacrylamide gels with 1.5% low melting agarose (BioLine GmbH, Luckenwalde, Germany) and traces of bromphenol blue. Electrophoresis was performed with constant voltage (20 V for 1 h; 120 V for 15 h) at 10 °C.

The gels were then stained with colloidal Coomassie Blue staining solution (10% ammonium sulfate, 0.12% Coomassie Brilliant Blue G-250 (AppliChem), 10% phosphoric acid, 20% methanol (Merck KGaA) (28)), and thereafter destained by washing in double distilled H₂O. All gels were scanned (UMAX Image Scanner, GE Healthcare) at a resolution of 600 dpi and stored as TIFF-images.

Two-dimensional gel image analysis was performed using Delta2D software version 4.0.8 (DECODON GmbH, Greifswald, Germany). All gel images were matched with the Delta2D software, and a synthetic fusion gel was prepared. Final spot detection was performed on the fused gel. The resulting spot pattern was assigned to each of the gels in the experiment. Student's t test was performed to assess the statistical significance of differentially expressed proteins. Based on average spot volume ratio, spots whose relative expression is changed at least 2-fold (increase or decrease) at 95% confidence level (t test; p < 0.05) were considered to be significant and subsequently subjected to further analysis.

In-gel Digestion and MS—Digestion with trypsin and subsequent spotting of peptide solutions onto the MALDI targets were performed as described previously (19) with slight modifications. For protein identification, the proteins were excised from stained two-dimensional gels using the Herolab spot hunter (Herolab GmbH, Wiesloch, Germany) and destained by addition of 50% (v/v) ACN. Gel pieces were washed twice with 100 µl of 50% (v/v) ACN and once with 100 µl of 100% ACN. After drying, 5 µl of trypsin solution containing 17 ng/µl trypsin (Promega, Madison, WI) in 25 mM NH₄CO₃ supplemented with 0.4 mM CaCl₂ was added and incubated on ice for 2 h followed by an incubation overnight at 37 °C. If necessary, 5 µl of 25 mM NH₄CO₃ supplemented with 0.4 mM CaCl₂ was added to keep gel pieces hydrated throughout the digest. Gel pieces were agitated by sonication in a water bath for 10 min before 1 µl of the supernatant (containing tryptic peptides) was mixed with 1 µl of α-cyano-4-hydroxycinnamic acid matrix (saturated at room temperature in 50% ACN, 0.1% TFA), and 1 µl of this solution was directly spotted on the MALDI target and allowed to dry.
Spectra were calibrated externally using Bruker’s peptide calibration standard II (Bruker Daltonics Inc., Bremen, Germany). MALDI-TOF-MS was performed on an ultrafleXtreme™ mass spectrometer (Bruker Daltonics Inc.) in positive reflector mode using an accelerating voltage of 25 kV. Spectra processing was performed with FlexAnalysis (3.3.80.0) software for resolution-based peak detection using default settings.

The PMF dataset were analyzed using the MASCOT search engine with the following parameters: (i) Homo sapiens sequences; (ii) fixed modification, carbamidomethylation of cysteines; (iii) cleavage enzyme, trypsin; (vi) a maximum of one missed cleavage was allowed; and mass tolerance (monoisotopic) was ± 50.0 ppm. Target identification was based on the overall sequence coverage of matching peptide fragments. Proteins were assigned when the MASCOT score exceeded 57 overall sequence coverage of matching peptide fragments. Proteins were assigned when the MASCOT score exceeded 57 overall sequence coverage of matching peptide fragments. The heterogeneous set of the identified significant differentially expressed proteins was analyzed using gene ontology (GO miner software (29)), which provides information about gene function and cellular localization. The results are listed in Table 1.

### Table 1: Differentially expressed proteins upon miR-152 overexpression in JEG-3 cells determined by 2DE-based proteomic profiling and mass spectrometry

| Spot no. | Protein name | Official gene symbol | Protein accession no. | Sequence coverage | Mascot score | x-Fold change | Cellular function | Cellular compartment |
|----------|--------------|----------------------|-----------------------|-------------------|-------------|--------------|-------------------|---------------------|
| 02       | Thiorexin    | TXN                  | P10599                | 80                | 106         | 2.2          | Electron transport; transcription regulation | Cytoplasm, nucleus, secreted |
| 04       | Nucleoside diphosphate kinase A | NME1 | P15531                | 69                | 94          | 2.3          | Protein biosynthesis | Cytoplasm |
| 05       | Elongation factor 2 | EEF2 | P13639                | 31                | 151         | 13.2         | Cell shape | Cytoplasm |
| 06       | Ezrin        | EZR                  | P15311                | 34                | 172         | 2.3          | | Cell membrane, cell projection, cytoplasm, cytoskeleton, membrane |
| 07       | Endoplasm    | HSP90B1              | P14625                | 22                | 132         | 0.5          | Chaperone | Endoplasmic reticulum |
| 08       | Fascin       | FSCN1                | Q16658                | 48                | 176         | 2.0          | Actin-binding, cell migration, cell proliferation | Cell junction, cell projection, cytoplasm, cytoskeleton |
| 10       | Keratin, type II cytoskeletal 8 | KRT8 | P05787                | 48                | 195         | 0.5          | Cytoskeleton organization | Cytoplasm, intermediate filament, keratin, nucleus |
| 11       | 60-kDa heat shock protein, mitochondrial | HSPD1 | P10809                | 34                | 140         | 0.4          | Mitochondrial protein import and macro-molecular assembly | Mitochondrion |
| 12       | Heat shock protein HSP 90-α | HSP90AA1 | P07900                | 18                | 96          | 0.3          | Stress response | Cytoplasm |
| 13       | Multifunctional protein ADE2 | PAICS | P22234                | 28                | 93          | 2.5          | Purine biosynthesis | Cytoplasm |
| 14       | α-Enolase    | ENO1                 | P06733                | 41                | 115         | 0.4          | Glycolysis, plasminogen activation, transcription regulation | Cell membrane, cytoplasm, membrane, nucleus |
| 15       | Eukaryotic translation initiation factor 2 subunit 1 | EIF2S1 | P05198                | 35                | 105         | 0.3          | Protein biosynthesis, translation regulation | Cytosol, nucleus |
| 16       | Annexin A2   | ANXA2                | P07355                | 36                | 102         | 2.7          | Calcium-regulated membrane-binding protein, angiogenesis, heat-stress response | Basement membrane, extracellular matrix, secreted |
| 17       | Protein-disulfide isomerase A6 | PDA6 | Q15084                | 49                | 180         | 0.2          | Chaperone, isomerase | Cell membrane, endoplasmic reticulum |
| 18       | 26 S proteasome non-ATPase regulatory subunit 14 | PSMD14 | O00487                | 46                | 106         | 2.4          | DNA damage, DNA repair, Ubl conjugation pathway | Proteasome |
| 19       | Calreticulin | CALR                 | P27797                | 24                | 65          | 2.8          | Chaperone | Cytoplasm, endoplasmic reticulum, extracellular matrix, sarcoplasmic reticulum, secreted |
| 20       | Proliferating cell nuclear antigen | PCNA | P12004                | 68                | 145         | 0.4          | DNA damage, DNA repair, DNA replication | Nucleus |
| 21       | Elongation factor Tu, mitochondrial | TUFM | P49411                | 44                | 165         | 0.4          | Protein biosynthesis | Mitochondrion |
| 22       | Protein-disulfideisomerase A3 | PDIA3 | P30101                | 25                | 107         | 0.4          | Isomerase | Endoplasmic reticulum |
| 23       | 14-3-3 protein ε | YWHAE | P62258                | 47                | 102         | 0.5          | Adapter protein, regulation of signaling pathways | Cytoplasm |
| 24       | 14-3-3 protein β/α | YWHAB | P31946                | 31                | 74          | 0.4          | Adapter protein, regulation of signaling pathways | Cytoplasm |
| 25       | Growth factor receptor-bound protein 2 | GRB2 | P62993                | 36                | 63          | 0.4          | Adapter protein, link between cell surface growth factor receptors and the Ras signaling pathway | Cytoplasm, endosome, Golgi apparatus, nucleus |
| 26       | ESI protein homolog, mitochondrial | C21orf33 | P30042                | 45                | 90          | 0.4          | | Mitochondrion |
| 27       | Keratin, type I cytoskeletal 19 | KRT19 | P08727                | 38                | 152         | 0.5          | Organization of myofibers | Intermediate filament, keratin |

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Semi-quantitative and Quantitative PCR—cDNA was synthesized from 2 μg of total cellular RNA using random hexamer primers (Fermentas, Mannheim, Germany) and the ReverTaid™ H Minus First Strand cDNA synthesis kit according to the manufacturer’s instructions (Fermentas, St. Ingbert, Germany). For miR-specific cDNA synthesis, miR-specific stem-loop primers altered after Chen et al. 2005 (21) were employed (22). PCR was performed with target-specific primers using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen). The reverse transcription and PCRs were carried out in a 96-well labycler (Sensoquest, Goettingen, Germany); the qPCRs for the quantification of miRs were performed in a Bio-Rad 96-well iCycler (Bio-Rad), and the relative quantification of mRNAs was determined in a rotor gene cycler (Qiagen). All reactions were run as triplicates of biologic replicates. For qPCR of miRs, the absolute copy numbers were determined against the respective external miR-specific TOPO-TA plasmid standards (Invitrogen), which were generated by cloning the stem-loop PCR product of the miR of interest into this plasmid. Relative mRNA expression levels for specific genes were normalized to GAPDH. All oligonu-
cleotides used for the mRNA and miR expression profiling are listed in Table 2.

For analysis of the gene expression involved in regulation of cell cycle and apoptosis, cells were synchronized by serum starvation (0.5% FCS (v/v)) for 48 h, and the cells were then cultured for 48 h in complete medium (10% FCS) before harvesting for RNA extraction.

**Protein Extraction and Western Blot Analysis**—Protein extraction and Western blot analyses were performed as described elsewhere (23). 50 or 70 μg of protein/lane were separated on 12% or 14% SDS-polyacrylamide gels and subsequently transferred onto nitrocellulose membranes (Schleicher & Schuell). Membranes were processed as described previously (23) using target protein-specific primary antibodies directed against 14-3-3-8 (polyclonal antibody C-20; Santa Cruz Biotechnology) and the monoclonal antibodies (mAb) directed against HLA-G (polyclonal antibody C-20; Santa Cruz Biotechnology), BAX, and cleaved caspase 3 (Cell Signaling) in combination with horse-radish peroxidase (HRP)-conjugated secondary antibodies (DAKO, Hamburg, Germany). Staining with an anti-GAPDH antibody (Cell Signaling) served as loading control, and the relative protein expression level for each target was defined using

### Table 2

Oligonucleotides applied in this study

| Primer        | Application          | Sequence (5' → 3')                                                                 | Condition |
|---------------|----------------------|-----------------------------------------------------------------------------------|-----------|
| miR-141       | Stem-loop primer     | GCCTTACCAATTTGTACCAGG                                                                 | 58 °C     |
| miR-141 fw    | qPCR                 | GCCTTACCAATTTGTACCAGG                                                                 | 60 °C     |
| miR-152       | Stem-loop primer     | GCCTTACCAATTTGTACCAGG                                                                 | 58 °C     |
| miR-152 fw    | qPCR                 | GCCTTACCAATTTGTACCAGG                                                                 | 60 °C     |
| miR-541       | Stem-loop primer     | GCCTTACCAATTTGTACCAGG                                                                 | 58 °C     |
| miR-541 fw    | qPCR                 | GCCTTACCAATTTGTACCAGG                                                                 | 60 °C     |
| Stem loop PCR  | rev primer           | GTCCAGTCTCCAGG                                                                      | 60 °C     |
| Clone miR-152 | Cloning              | AAACTGCGCTGGTCTTGGGGGTAG                                                          | 60 °C     |
| Clone miR-152 | rev Cloning          | AAACTGCGCTGGTCTTGGGGGTAG                                                          | 60 °C     |
| Clone miR-541 | Cloning              | AAACTGCGCTGGTCTTGGGGGTAG                                                          | 60 °C     |
| Clone miR-541 | rev Cloning          | AAACTGCGCTGGTCTTGGGGGTAG                                                          | 60 °C     |
| Clone YWHAB   | Cloning              | AAACTGCGCTGGTCTTGGGGGTAG                                                          | 58 °C     |
| Clone YWHAB   | rev Cloning          | AAACTGCGCTGGTCTTGGGGGTAG                                                          | 58 °C     |
| qPCR           | Primers              | qPCR                                                                               | 60 °C     |
| HLA-GmiTrAP   | fw Cloning           | AAACTGCGCTGGTCTTGGGGGTAG                                                          | 60 °C     |
| HLA-GmiTrAP   | rev Cloning          | AAACTGCGCTGGTCTTGGGGGTAG                                                          | 60 °C     |
| miTrAP YWHAB  | Cloning              | AAACTGCGCTGGTCTTGGGGGTAG                                                          | 58 °C     |
| luc YWHAB     | Cloning              | AAACTGCGCTGGTCTTGGGGGTAG                                                          | 58 °C     |
| luc YWHAB     | rev Cloning          | AAACTGCGCTGGTCTTGGGGGTAG                                                          | 58 °C     |
| Δ15291 fw     | Fusion PCR           | AAACTGCGCTGGTCTTGGGGGTAG                                                          | 58 °C     |
| Δ15291 rev    | Fusion PCR           | AAACTGCGCTGGTCTTGGGGGTAG                                                          | 58 °C     |
| qPCR           | CDK1 fw              | qPCR                                                                               | 60 °C     |
| qPCR           | CDK1 rev             | qPCR                                                                               | 60 °C     |
| qPCR           | CDK2 fw              | qPCR                                                                               | 60 °C     |
| qPCR           | CDK2 rev             | qPCR                                                                               | 60 °C     |
| qPCR           | CDK4 fw              | qPCR                                                                               | 60 °C     |
| qPCR           | CDK4 rev             | qPCR                                                                               | 60 °C     |
| qPCR           | CDC6 fw              | qPCR                                                                               | 60 °C     |
| qPCR           | CDC6 rev             | qPCR                                                                               | 60 °C     |
| qPCR           | CCNB rev             | qPCR                                                                               | 60 °C     |
| qPCR           | CCNA fw              | qPCR                                                                               | 60 °C     |
| qPCR           | CCNA rev             | qPCR                                                                               | 60 °C     |
| qPCR           | CCND1 fw             | qPCR                                                                               | 60 °C     |
| qPCR           | CCND1 rev            | qPCR                                                                               | 60 °C     |
| qPCR           | CCNE fw              | qPCR                                                                               | 60 °C     |
| qPCR           | CCNE rev             | qPCR                                                                               | 60 °C     |
| qPCR           | BAX fw               | qPCR                                                                               | 60 °C     |
| qPCR           | BAX rev              | qPCR                                                                               | 60 °C     |
| qPCR           | BAD fw               | qPCR                                                                               | 60 °C     |
| qPCR           | BAD rev              | qPCR                                                                               | 60 °C     |
| qPCR           | BCL2 fw              | qPCR                                                                               | 60 °C     |
| qPCR           | BCL2 rev             | qPCR                                                                               | 60 °C     |
| qPCR           | Survivin fw          | qPCR                                                                               | 60 °C     |
| qPCR           | Survivin rev         | qPCR                                                                               | 60 °C     |
| qPCR           | McI1 fw              | qPCR                                                                               | 60 °C     |
| qPCR           | McI1 rev             | qPCR                                                                               | 60 °C     |
| qPCR           | Pten fw              | qPCR                                                                               | 60 °C     |
| qPCR           | Pten rev             | qPCR                                                                               | 60 °C     |
| qPCR           | TP53 fw              | qPCR                                                                               | 65 °C     |
| qPCR           | TP53 rev             | qPCR                                                                               | 65 °C     |

For the full text, please refer to the original source.
AIDA software (Raytest, Sprockhoevel, Germany). Protein bands were visualized with the Lumi-Light Western blotting substrate (Roche Applied Science) and recorded with an LAS 3000 CCD camera system (FUJIFILM, Düsseldorf, Germany).

Flow Cytometry—Flow cytometry was performed for analysis of cell proliferation and apoptosis induction using 5,6-carboxyfluorescein diacetate-succinimidyl ester (CFSE) staining (Invitrogen) and allo-phycocyanine-conjugated annexin V (Pharmingen) in combination with 7-aminactinomycin D staining (Pharmingen), respectively, according to the manufacturer’s instructions as recently described (24). To evaluate cell proliferation, 3 × 10⁶ cells were labeled with CFSE for 15 min at 37 °C in 10 ml of phosphate-buffered saline (PBS) supplemented with 0.3% FCS, according to the manufacturer’s instructions, and finally seeded in 6-well microtiter plates (Techno Plastic Products AG, TTP, Trasadingen, Switzerland). After 24 h, the mean specific fluorescence intensity of the CFSE-labeled cells was analyzed before the cells were treated with 25 nM paclitaxel (Taxol®) or DMSO in a time kinetic fashion in the absence of any antibiotics. After 24, 48, and 72 h of treatment, cells were analyzed by flow cytometry.

Cell cycle analysis was performed with synchronized cells as described above. Nuclei were isolated by incubation of cells in 10 mM citric acid supplemented with 0.5% (w/v) Tween 20 for 30 min at 4 °C in 1 ml of phosphate-buffered saline (PBS) supplemented with 0.3% FCS, according to the manufacturer’s instructions, and finally seeded in 6-well microtiter plates (Techno Plastic Products AG, TTP, Trasadingen, Switzerland). After 24 h, the mean specific fluorescence intensity of the CFSE-labeled cells was analyzed before the cells were treated with 25 nM paclitaxel (Taxol®, Bristol-Myers Squibb, New York) or DMSO in a time kinetic fashion in the absence of any antibiotics. After 24, 48, and 72 h of treatment, cells were analyzed by flow cytometry.

Viability Assay—To determine the viability of the transfected cells upon treatment with paclitaxel, an 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay was performed according to the manufacturer’s instructions (cell proliferation kit II, Roche Applied Science) using 3000 cells/well in 150 µl of media. Cell viability was analyzed after incubation of cells for 72 h in the absence and presence of paclitaxel (0–20 µM) using a microplate reader (MRX-TC, DYNEX Technologies, Denkendorf, Germany). The absorbance values were expressed as a percentage of the DMSO-treated control group, and IC₅₀ values were calculated as published by Stehle et al. (24).

Generation of 14-3-3β and miR Expression Vectors and Cell Transfection—For generation of the 14-3-3β expression vector, cDNA from JEG-3 cells was utilized as template for PCR (primer sequences listed at Table 2). The resulting PCR product was cloned into the pMiR-REPORT vector (Ambion, Austin, TX) by restriction with BamH I and MluI (Fermentas) replacing the luciferase (luc) gene. The miR genes and their flanking regions were cloned into the pMIR-m-cherry vector (Clontech) using the restriction enzymes Xho I and EcoRI (Fermentas). JEG-3 cells were stably transfected with the miR, the 14-3-3β expression vector, and the respective mock vector controls, using the Effectene transfection reagent (Qiagen; Hilden, Germany) according to the manufacturer’s protocol.

Luciferase Reporter Gene Assay and Cell Transfection—For the luc reporter gene assays, the 3′-UTR of 14-3-3β was inserted downstream of the luc reporter gene into the pMiR Report vector (Invitrogen) using the restriction enzymes SpeI and MluI (New England Biolabs). To determine the specificity of miR and target interaction, the respective miR-binding site was deleted within the luc reporter gene construct by fusion PCR. To block miR-152, a respective decoy vector was generated according to Haraguchi et al. (25) by hybridization and cloning of complementary oligonucleotides (Table 2) into the pLVX-iRES-ZsGreen (Clontech).

For transfection, 1 × 10⁴ HEK293T cells were seeded into flat bottom 96-well plates, incubated for 24 h at 37 °C, and then transfected with either the miR expression vector or the mock control using the Effectene transfection reagent (Qiagen). After 24 h of incubation at 37 °C, a second transfection was performed using the luc reporter gene constructs and the β-galactosidase (β-gal) vector, the latter serving as control for normalization of transfection efficacy. 72 h after seeding, the cells were harvested with lysis buffer (Promega, Madison, WI). The luc and β-gal activities were determined with a luminometer (Microlumat Plus CB 96V, Berthold Technologies, Bad Wildbad, Germany) using commercially available enzyme assays (Promega) as described recently (23). The results were expressed as a quotient of the luc and β-gal activities.

miR Enrichment Assay (miTRAP)—The miTRAP technology has recently been established and described in detail (26). Briefly, the 3′-UTRs of 14-3-3β and HLA-G were cloned upstream of a sequence encoding for four MS2 loops using the restriction enzymes Xho I and EcoRI (Fermentas), in vitro transcribed with riboprobe (Promega), purified with the MEGAClear™ kit (Ambion), and then used for the enrichment of specific miRs from MZ2905RC cell lysate (HLA-G mRNA+/protein−) (7)). First, 500 pmol of fusion protein consisting of MS2 loop and maltose-binding protein domains was coupled to amylose beads (Amylose Resin; New England Biolabs) and purified as described elsewhere (27). After washing, a blocking step with bovine serum albumin (New England Biolabs) and yeast tRNA (Promega) was performed. Then after washing, 20 pmol of in vitro transcribed RNA were employed as bait/pulldown and loaded onto the beads. After washing, the beads were incubated with 500 µl of cell lysate (equal to lysate of 3.5 × 10⁶ cells) followed by extensive washing and RNA extraction. The miR enrichment was validated by respective qPCR. RNA extraction and cDNA synthesis were performed as described by Braun et al. (26). The principle of this novel experiment is shown in Fig. 8A. The usage of terminal MS2 loops ensures the formation of
the secondary structure of the upstream 3′-UTR RNA sequence to guarantee the correct interactions with RNA-binding proteins and miRs.

**Analysis of Clinical cDNA Microarrays for Detection of Correlations between 14-3-3β, HLA-G, and Patient Survival—** Transcriptome data obtained from patient samples of various cancer types have been analyzed using the free on-line database R2, microarray analysis, and visualization platform to correlate the expression of 14-3-3β (YWHAB) and HLA-G with the overall survival of tumor patients.

**RESULTS**

**Reconstitution of miR-152 Expression in JEG-3 Cells, Establishment of the Model System—** The expression pattern of the HLA-G-specific candidate miR-152 and miR-141, which served as an internal control, was determined in HLA-G⁺ JEG-3 cells and different RCC and melanoma cell lines. The choriocarcinoma cell line JEG-3, but not the other cell lines tested, expressed low levels of miR-152 (Fig. 1A). This was in line with high levels of HLA-G protein in JEG3 cells, whereas the RCC and melanoma cells tested lack HLA-G expression (4, 16). Furthermore, miR-152 expression was restored in JEG-3 cells by stable transfection of an miR-152 expression plasmid resulting in ~1000-fold increase of the miR-152 copy number (Fig. 1B), which is physiologic and comparable with the transcripts found in HLA-G⁺ tumor cells. This was accompanied by a loss of HLA-G protein expression (Fig. 1C). The miR-152-induced downregulation of HLA-G expression proves the functionality of the overexpressed miR-152. In contrast, miR-141 expression used as internal control remained unaffected in miR-152high cells (Fig. 1B).
Identification of Novel miR-152 Targets by Proteome Analysis—
To determine novel targets of miR-152, the protein expression pattern of mock-transfected (miR-152low) and miR-152-transfected JEG-3 cells (miR-152high) was compared using 2DE-based proteome analysis. Differentially expressed protein spots of miR-152low versus miR-152high model systems were then subjected to mass spectrometric analysis. As listed in Table 1, 24 proteins were differentially expressed upon miR-152 overexpression, from which 14 proteins were down-regulated and 10 proteins were up-regulated in miR-152low versus miR-152high JEG-3 cells. Regarding their classification, these proteins are mainly involved in metabolism and biogenesis (Table 1 and Fig. 7). Because 14-3-3 proteins are associated with oncogenic features (30) that could explain the observed differences in proliferation upon miR-152 overexpression (described below), further studies focused on the expression, function, and regulation of 14-3-3β by miR-152.

Validation of 14-3-3β as a Specific miR-152 Target—
The strong miR-152-mediated down-regulation of 14-3-3β mRNA and protein expression was validated by qPCR (Fig. 2, A and B) and Western blot analysis of miR-152low versus miR-152high JEG-3 and Buf1088 cells. It is noteworthy that the 14-3-3β protein is heterogeneously expressed in the different (tumor) cells analyzed. Although it was not detectable or only barely detectable in the melanoma cell line FM82 or in the RCC cell lines, respectively, the highest expression levels of 14-3-3β protein were found in Buf1088 and WM1862 cells (Fig. 2C). Because only JEG-3 cells exert a miR-152 deficiency, a correlation between miR-152 and 14-3-3β protein in these cells could not be observed. In this context, it is noteworthy that 14-3-3β down-regulation might be also attributed to other mechanisms, such as epigenetic silencing, mutation, or dysregulation of p53 or its rapid degradation due to its ubiquination as reported for 14-3-3σ (31, 32).

Direct Interaction between miR-152 and the 14-3-3β 3′-UTR—
In silico analyses of the miR-152 binding to the target mRNA of 14-3-3β were performed demonstrating that miRanda, miRDB, miRwalk, TargetScan, and RNA hybrid predict a miR-152-binding site (33–36). To further study the interaction between miR-152 and 14-3-3β, a fragment of the 14-3-3β 3′-UTR, including the in silico predicted miR-152-binding site, was cloned behind the luc reporter gene. A deletion (Δ) construct lacking the in silico predicted miR-152-binding site served as control (Fig. 3D). These constructs were co-transfected with the miR expression vectors pmR(mock), pmR(miR-152), and pmR(miR-541) as nonsense control, which so far only affects neuronal differentiation (37). As shown in Fig. 3A, a significantly reduced luc activity was detected by co-transfection with the miR-152 expression vector. However, this inhibition was completely abolished by deleting the in silico predicted miR-152-binding site in the reporter gene construct.

To further investigate the effect of miR-152 on the 3′-UTR of 14-3-3β, this miR was inhibited by a miR-152 decoy construct. By blocking miR-152, the luc activity in combination with the 3′-UTR of 14-3-3β was significantly stabilized when compared with the respective mock controls (Fig. 3B).
Furthermore, the wild type (WT) 3'-UTRs of 14-3-3β and HLA-G were employed for miTRAP to enrich miRs from the HLA-G mRNA+/-HLA-G protein− RCC MZ2905RC cell line. The principle of the miTRAP experiment is shown in Fig. 8. Using the HLA-G 3'-UTR as bait (positive control), miR-152 could be enriched, whereas miR-141 (internal negative control) was not enriched due to the lack of a binding site in the 3'-UTR of HLA-G and of 14-3-3β. Interestingly, the enrichment of miR-152 using the 14-3-3β WT 3'-UTR was 10 times higher when compared with that of the HLA-G 3'-UTR (Fig. 3C).

**Altered Growth Properties and Apoptosis Sensitivity of 14-3-3β Transfectants**—Because 14-3-3β could exert anti-apoptotic activity, promote tumor proliferation, and decrease overall survival of tumor patients (11, 38–41), the impact of an altered 14-3-3β expression in the miR-152hish JEG-3 cells (miR-152-overexpressing transfectants) was determined. Therefore, the
down-regulated 14-3-3β expression was reconstituted by stable overexpression of the 14-3-3β coding sequence without the 3'-UTR into miR-152high JEG-3 cells leading to an ~3-fold increase in 14-3-3β transcript (Fig. 4A) and protein levels (Fig. 4B). The miR-152-mediated down-regulation of the 14-3-3β expression upon miR-152 overexpression resulted in an up-regulation of the pro-apoptotic protein BAX, whereas the level of cleaved caspase 3 and the content of early apoptotic cells remained unaffected (Fig. 4, C and E) in untreated cells. In contrast, treatment with 25 nM paclitaxel for 72 h reduced the level of pro-apoptotic BAX within the 14-3-3β-expressing rescue variant (Fig. 4C). Furthermore, treatment with 25 nM paclitaxel for 72 h increased the number of early apoptotic cells about 50% in both miR-152low and miR-152high cells, although overexpression of the anti-apoptotic 14-3-3β rescue variant lacking the 3'-UTR significantly reduced apoptosis sensitivity (Fig. 4E).

The reconstitution of the 14-3-3β protein in the miR-152high cells resulted in a significant down-regulation of apoptosis, which could be explained by the detected up-regulation of the transcription level of the anti-apoptotic BCL2 gene (Fig. 4F). The presence of paclitaxel alone already induced the up-regu-
lation of BCL2 gene expression, but this effect was strongly increased upon 14-3-3β reconstitution.

To address the clinical relevance of the 14-3-3β-mediated effects on the apoptosis, the transfectants with the highest difference in the 14-3-3β expression (miR-152high + pExp(mock) and miR-152high + pExp(14-3-3β)) were treated with different concentrations (0–20 μM) of the chemotherapeutic drug paclitaxel.

The cytotoxic effects were monitored at different drug concentrations to define the respective 50% proliferation/growth inhibition concentration (IC50) values via XTT assays. As shown in Fig. 4D, a dose-dependent but incomplete inhibition of cell viability was detected in the presence of paclitaxel.

However, upon drug treatment there were no significant changes within the IC50 values for paclitaxel (3.2 ± 0.5 nM), but the residual metabolic activity increased from 52 ± 3 to 62 ± 3% in the presence of paclitaxel when compared with the miR-152high/14-3-3βlow cells (Fig. 4D). Furthermore, high expression levels of 14-3-3β could be correlated to a poor overall survival of patients of distinct cancer diseases (Fig. 6).

In coincidence with the previously described function as a tumor suppressor, the miR-152-mediated down-regulation of 14-3-3β decreased the proliferation rate in the presence of 25 nM paclitaxel at an average of about 11%, whereas the expression of the 14-3-3β rescue variant enhanced the proliferative
capacity of these cells from 59 to 75% significantly as measured by flow cytometry after CFSE labeling and incubation for 72 h in the presence of 25 nM paclitaxel (Fig. 5A). In the absence of the chemotherapeutic drug, no differences on the cell proliferation capacity (85 to 87% proliferating cells) were detected. To further characterize the proliferation rate upon 14-3-3\(\beta\)/H9252 protein overexpression, cell cycle analysis of the JEG-3 transfectants miR-152\textsubscript{high}/H11001 pExp (mock) and miR-152\textsubscript{high}/H11001 pExp(14-3-3\(\beta\)) was performed.

As shown in Fig. 5B, the 14-3-3\(\beta\) reconstitution resulted in a significantly increased number of mitotic cells in miR-152-overexpressing JEG-3 cells (miR-152\textsubscript{high} + pExp(14-3-3\(\beta\)). In addition, the expression of genes involved in cell cycle regulation was analyzed by qPCR.

Although cyclin B and the cyclin-dependent kinases 1 and 4 showed no differential expression at the transcript level, gene expression of cyclin E and cyclin-dependent kinase 2 were significantly up-regulated upon 14-3-3\(\beta\) protein expression,
therefore enhancing $G_1$ to $S$ cell cycle phase transition, determining the cell division. In contrast, cyclin A and D and cyclin-dependent kinase 6 were down-regulated (Figs. 5C and 8C).

Correlation of 14-3-3β and HLA-G Expression with Patient Survival—The expression of both HLA-G and 14-3-3β is controlled by miR-152. Whereas HLA-G allows tumors to escape the immune system, 14-3-3β decreases the sensitivity of tumor cells to apoptosis (Fig. 6) (38 – 40, 42). To determine whether the expression of both genes could be correlated with clinical parameters, in vivo cDNA microarrays were analyzed using patients’ data from various cancer entities (neuroblastoma, lung cancer, glioma, and osteosarcoma) obtained from the R2 on-line database. Unfortunately, no RCC or melanoma data sets were available in the database. However, the patients’ cohorts characterized by high HLA-G or high 14-3-3β mRNA expression levels revealed a significantly decreased overall survival of patients when compared with that characterized by low expression levels of both genes. As illustrated in Fig. 5, the expression and the survival data of these two miR-152 targets could be directly correlated, thereby strengthening the prognostic potential of HLA-G, 14-3-3β, and miR-152 expression in various cancer types.

**DISCUSSION**

In this study, a novel miR-152 target, the protein 14-3-3β, was identified by comparative 2DE-based proteomic profiling of miR-152-overexpressing transfectants and control cells. Because the choriocarcinoma cell line JEG-3 expressed very low levels of miR-152, this miR was stably overexpressed in JEG-3 cells, which resulted in a down-regulation of the known miR-152 target HLA-G (4), thereby demonstrating the functionality of the model system.

Recently, 2DE-based proteomics has been employed as a strategy to identify novel miR targets (43). One advantage of proteome-based methods for miR target identification compared with transcriptome-based technologies is the readout at the protein level, because miRs must not induce mRNA decay but miR binding leads to translational repression of the target mRNAs (44).

The comparison of the protein expression patterns of mock- and miR-152-transfected JEG-3 cells (miR-152low versus miR-152high) led to the identification of 24 differentially expressed proteins, which are mainly involved in cellular metabolism and biogenesis (Table 1 and Fig. 7) As miR-152 was shown to be a tumor-suppressive miR, and its expression is associated with a decreased cell proliferation and an increased apoptosis (11–14), the following studies focused on the novel anti-apoptotic miR-152 target 14-3-3β, a phosphoserine/phosphothreonine-binding protein that was down-regulated upon miR-152 expression. The highly conserved 14-3-3 protein family plays a key role in various cellular processes, such as metabolism, protein trafficking, signal transduction, apoptosis, and cell cycle regulation. Because many interactions of 14-3-3 members with other proteins are phosphorylation-dependent, 14-3-3β has been tightly integrated into the core phospho-regulatory pathways that are crucial for normal growth and development and often become deregulated in human malignancies, including cancer (45). 14-3-3β exerts anti-apoptotic activity, promotes tumor prolif-

![FIGURE 7. Annotation clustering of the differentially expressed proteins upon miR-152 overexpression in JEG-3 cells identified by 2DE-based proteomic profiling and mass spectrometry.](image-url)
14-3-3β exerts anti-apoptotic activities thereby enhancing the viability of the respective transfectants upon treatment with the chemotherapeutic drug paclitaxel due to a significant up-regulation of BCL2. This is in line with an increased proliferation of tumor cells by up-regulation of the CCNE-CDK2 complex. The analysis of patients surviving certain tumor entities demonstrates a direct inverse correlation of the 14-3-3β expression levels with the overall survival rate of tumor patients. Furthermore, these data are in line with a high frequency of 14-3-3β overexpression in human tumors of distinct origin, which is also often accompanied by a reduced apoptosis sensitivity.

The direct interaction of 14-3-3β and miR-152 was characterized using luc reporter gene analyses and with the miR-152 blocking experiment and miR enrichment assay (miTRAP). Using a region of the 3’-UTR of 14-3-3β (2414–3067 bp of the mRNA sequence) containing the in silico predicted miR-152-binding site as bait for the specific miRNA enrichment assay (miTRAP) revealed a specific enrichment of miR-152 from MZ2905RC cell lysate (Fig. 3B). Functional studies further demonstrated an anti-apoptotic activity of 14-3-3β, which might also have clinical relevance and might serve as a prognostic factor for tumor patients.
Dual Role of miR-152

The 14-3-3 proteins dimerize to homo- and heterodimers, which interact with a large number of cellular proteins, including transcription factors, cytoskeletal proteins, signaling molecules, apoptosis factors, and tumor suppressors (38, 46, 47). Factors involved in apoptosis, which were inhibited by 14-3-3 proteins, are the pro-apoptotic proteins BAX- and BCL2-associated agonist of cell death (BAD) (39, 40). These data are in line with the miR-152-mediated down-regulation of 14-3-3β, which resulted in increased BAX protein levels (Fig. 4C) as well as in decreased BCL2 gene expression (Fig. 4F). Furthermore, the decreased sensitivity toward the chemotherapeutic drug paclitaxel after reconstitution of the decreased sensitivity toward the chemotherapeutic drug paclitaxel after reconstitution of the decreased sensitivity toward the chemotherapeutic drug paclitaxel after reconstitution of the decreased sensitivity toward the chemotherapeutic drug paclitaxel after reconstitution of the decreased sensitivity toward the chemotherapeutic drug paclitaxel after reconstitution of the decreased sensitivity toward the chemotherapeutic drug paclitaxel after reconstitution of the decreased sensitivity toward the chemotherapeutic drug paclitaxel after reconstitution of the decreased sensitivity toward the chemotherapeutic drug paclitaxel after reconstitution of the decreased sensitivity toward the chemothera-

In addition, analysis of cDNA microarrays with known clinical parameters, including patients’ survival data, revealed that 14-3-3β and HLA-G share a common regulation mechanism, and their high expression levels could be correlated with a poor overall survival of patients with tumors. The miR-152-mediated regulation of both 14-3-3β and HLA-G links the HLA-G-induced immune escape mechanism with 14-3-3β-associated transformed growth properties of tumors (Fig. 8B).

In accordance with these data, 14-3-3β and -ζ have been demonstrated to be often overexpressed in human tumors of distinct origin and have been suggested as clinically relevant prognostic biomarkers due to their association with tumor progression and poor patient survival (11, 48, 49). This might allow the identification of tumor patients with poor prognosis to receive a more aggressive treatment. Even more interesting, the expression levels of 14-3-3β were higher in urine samples from patients with RCC than in samples from healthy volunteers, and therefore 14-3-3β may be a diagnostically useful biomarker for early diagnosis of this disease (50).

In conclusion, this study not only extended previously identified possible direct targets of miR-152, like HLA-G, DNMT-1, E2F3, MET, and RICTOR, to 14-3-3β suggesting that its silencing contributes to tumorigenesis, but identified for the first time a dual role of miR-152 by modulating not only the growth characteristics of tumor cells but also immune surveillance (4, 12, 51). Thus, miR-148 family members might serve as potential diagnostic and prognostic markers and provide novel treatment strategy for tumors, which is in line with its postulated diagnostic and prognostic markers and provide novel treatment strategy for tumors, which is in line with its postulated diagnostic and prognostic markers and provide novel treatment strategy for tumors, which is in line with its postulated diagnostic and prognostic markers and provide novel treatment strategy for tumors, which is in line with its postulated diagnostic and prognostic markers and provide novel treatment strategy for tumors, which is in line with its postulated diagnostic and prognostic markers and provide novel treatment strategy for tumors, which is in line with its postulated diagnostic and prognostic markers and provide novel treatment strategy for tumors, which is in line with its postulated diagnostic and prognostic markers and provide novel treatment strategy for tumors, which is in line with its postulated diagnostic and prognostic markers and provide novel treatment strategy for tumors, which is in line with its postulated diagnostic and prognostic markers and provide novel treatment strategy for tumors, which is in line with its postulated diagnostic and prognostic markers and provide novel treatment strategy for tumors, which is in line with its postulated diagnostic and prognostic markers and provide novel treatment strategy for tumors, which is in line with its postulated diagnostic and prognostic markers and provide novel treatment strategy for tumors, which is in line with its postulated diagnostic and prognostic markers and provide novel treatment strategy for tumors, which is in line with its postulated diagnostic and prognostic markers and provide novel treatment strategy for tumors, which is in line with its postulated diagnostically useful biomarker.

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