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Effect of Wild-Type and Mutant E-Cadherin on Cell Proliferation and Responsiveness to the Chemotherapeutic Agents Cisplatin, Etoposide, and 5-Fluorouracil

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Key Words
E-cadherin mutations · Cell growth · Chemosensitivity

Abstract
Objectives: The cell adhesion molecule E-cadherin acts as a tumor and invasion suppressor and regulates cell proliferation. The aim of the present study was to investigate the impact of wild-type (wt) E-cadherin and tumor-derived mutant E-cadherin variants on the proliferation rate of MDA-MB-435S mammary carcinoma cells and the sensitivity of the cells to the chemotherapeutic drugs cisplatin, etoposide and 5-fluorouracil (5-FU) and whether p53 is involved in the chemotherapeutic response. ***Methods:*** Proliferation rate was measured by XTT cell viability assay in the presence or absence of chemotherapeutics. Chemosensitivity was also measured by colony formation assay. Expression of p53 was investigated by immunoblot analysis. The mutational hot spot region exon 5–8 of p53 was analyzed for mutations by denaturing high-performance liquid chromatography. ***Results:*** The growth rate of MDA-MB-435S cells transfected with wt E-cadherin was reduced as compared with the parental cell line. In contrast, tumor-associated mutations of exons 8 or 9 of the E-cadherin gene interfere with the growth-suppressive function of E-cadherin. Cisplatin sensitivity of wt and mutant E-cadherin-expressing MDA-MB-435S cells was reduced as compared with E-cadherin-negative, parental MDA-MB-435S cells. In contrast, chemosensitivity of parental, wt or mutant E-cadherin-expressing MDA-MB-435S cells measured after etoposide or 5-FU exposure was found to be similar in all tested cell lines. Since p53 influences the sensitivity of cells to chemotherapeutic agents, we investigated whether the p53 expression level or mutation status were different in the nontransfected or E-cadherin-transfected MDA-MB-435S cell lines. We found that the p53 expression pattern and genomic background were similar in all cell lines and not affected by cisplatin. ***Conclusion:*** The results obtained in this study suggest that the expression and/or mutation of the E-cadherin gene influence the proliferation rate and drug sensitivity of tumor cells.

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Introduction

E-cadherin, which is classically described as an invasion and tumor suppressor [1–3], acts also as a suppressor of cell growth, as demonstrated by cell culture and mouse experiments [4–10]. E-cadherin has been reported to inhibit cell proliferation by a mechanism which includes upregulation of the cyclin-dependent kinase inhibitor $p27^{KIP1}$ [11]. In another study, it has been shown that E-cadherin regulates cell growth by modulating the β-catenin transcriptional activity [10].

E-cadherin also plays a role in protecting cells from apoptosis [12–14]. The mechanism by which E-cadherin exerts its antiapoptotic function is not yet known. Kantak and Kramer [13] have suggested that interactions between E-cadherin and signaling molecules which are important for cellular survival are involved in the effect. For instance, E-cadherin has been shown to associate with the epidermal growth factor receptor which plays a role in cellular survival [15]. On the other hand, it has been suggested by Peluso [16] that the cytoplasmic E-cadherin binding partner β-catenin is involved in the antiapoptotic function of E-cadherin. The interaction of E-cadherin with the actin cytoskeleton is mediated by α- and β-catenin or plakoglobin [17, 18]. In addition to its role in cell adhesion, β-catenin is involved in the transcriptional regulation of the apoptosis-regulating genes c-*myc* [19] and c-*jun* [20].

The E-cadherin gene is frequently lost or mutated in tumors [21]. Somatic E-cadherin mutations were found in diffuse-type gastric and lobular breast carcinomas, comprising missense, splice site and truncation mutations [22–26]. Inactivating germline E-cadherin mutations have been identified in families with diffuse-type gastric carcinoma [27–30]. We have previously identified somatic E-cadherin mutations in 50% of sporadic diffuse-type gastric carcinoma [23]. Cloning and functional analysis of the tumor-associated E-cadherin mutations demonstrated that E-cadherin mutations influence regulatory cellular networks [31, 32]. E-cadherin mutations resulted in decreased cellular adhesion and increased cellular motility, alterations of the actin cytoskeleton, and an abnormal perinuclear localization of β-catenin.

Since our previous results suggest that E-cadherin mutations alter the functions of the wild-type (wt) molecule, we addressed the question of whether E-cadherin mutations have an impact on the growth-suppressive function of E-cadherin. Genetic alterations play also a causative role in tumor formation and progression. Specific genetic alterations, like E-cadherin mutations, might also determine the patient's outcome after chemotherapeutic treatment. Alterations in the expression level and functionality of E-cadherin are frequently observed in human cancer. To answer the question of whether the expression or mutation status of the E-cadherin gene influences chemosensitivity, the effect of cisplatin, etoposide and 5-fluorouracil (5-FU), all of which have been in clinical use for several years, on parental, wt and mutant E-cadherin-expressing MDA-MB-435S cells has been investigated.

Materials and Methods

Cells

The human mammary carcinoma cell line MDA-MB-435S (ATCC, Rockeville, Md., USA) and the E-cadherin-cDNA-transfected derivatives established by Handschuh et al. [31] were grown in Dulbecco's modified Eagle medium (DMEM, Life Technologies, Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS, PAN Biotech, Aidenbach, Germany) and penicillin-streptomycin (50 IU/ml and 50 μg/ml; Life Technologies) at 37 °C and 5% CO_2.

Reagents

Cisplatin (Sigma, Deisenhofen, Germany) was prepared as a 100 m*M* stock solution in DMSO. Etoposide (Calbiochem) was dissolved in DMSO as a 50 m*M* stock solution. 5-FU (Sigma) was prepared as a 100 mg/ml stock solution in DMSO.

XTT Cell Proliferation and Viability Assay

Cells were seeded at a density of 2×10^3 cells per well in 96-well microtiter plates (Nunc, Wiesbaden-Biebrich, Germany) in 100 μl DMEM with 10% FCS per well, and cell proliferation was investigated by XTT cell proliferation and viability assay (Roche Molecular Biochemicals, Mannheim, Germany). After 24, 48 and 72 h, 50 μl XTT labeling mixture was added. The cleavage of the tetrazolium salt XTT to form a formazan dye that occurs in metabolically active viable cells was quantified spectrophotometrically by measuring the absorbance of the formazan product at 450 nm by an ELISA plate reader. The absorbance values obtained when culture medium without cells was assayed was subtracted from the values obtained with cells. Quadruplicate determinations were performed for each time point.

Sensitivity Profiling by XTT Cell Proliferation and Viability Assay

The sensitivity of parental, wt and mutant E-cadherin-expressing MDA-MB-435S cells to the chemotherapeutic agents cisplatin, etoposide and 5-FU was investigated by XTT cell proliferation and viability assay as described above. Cells were seeded at a density of 2×10^3 cells per well in 96-well microtiter plates (Nunc, Wiesbaden-Biebrich, Germany) in 100 μl DMEM with 10% FCS per well and exposed to each drug for 48 h at various concentrations. After 48 h, 50 μl XTT labeling mixture was added and the absorbance values at 450 nm were measured spectrophotometrically by an ELISA plate reader. The absorbance values obtained when culture medium without cells was assayed were subtracted from the values obtained with cells. Quadruplicate determinations were performed for each time point. The percentage of viable cells was determined corresponding to nontreated cells.

Colony Formation Assay

Chemosensitivity to cisplatin, etoposide and 5-FU of parental, wt and mutant E-cadherin-expressing MDA-MB-435S cells was investigated by colony formation assay. Cells were seeded at a density of 2×10^3 cells per 6-well and treated 3 h later for 2 h with cisplatin, etoposide or 5-FU. Colonies were fixed and stained after 7 days with Diff Quick reagent (Dade Behring, Liederbach, Germany) and the colony number was determined with Scion Image Software from Scion Corporation (Frederick, Md., USA). The percentage of colonies was determined corresponding to nontreated cells.

p53 Mutation Analysis

Denaturing high-performance liquid chromatography (DHPLC), which uses heteroduplex formation between wt and mutant DNA to detect mutations, was performed according to the method of Oefner and Underhill [33] on an automated DHPLC analysis system (Transgenomic, Omaha, Nebraska). p53 mutation analysis was performed with DNA isolated from parental and transfected MDA-MB-435S cells using a DNA preparation kit (Qiagen, Hilden, Germany). Detection of p53 mutations in exon 5–8 by DHPLC was performed as described previously [34]. The primers and polymerase chain reaction (PCR) conditions for p53 sequence analysis were published by Keller et al. [34]. The purification of PCR products from agarose gels was performed with a gel extraction kit (Qiagen). For cycle sequencing, the Ready Reaction Big Dye Terminator Cycle Sequencing kit (Applied Biosystems) and an automated sequencing system (ABI 377, Applied Biosystems) were used.

Western Blot Analysis

For immunoblot analysis, cells were seeded at a density of 6×10^5 cells per 10-cm tissue culture dish, cultured in the presence or absence of 50 μ*M* cisplatin, and lysed 6 h later with 500 μl L-CAM buffer (140 m*M* NaCl, 4.7 m*M* KCl, 0.7 m*M* $MgSO_4$, 1.2 m*M* $CaCl_2$, 10 m*M* Hepes pH 7.4, containing 1% (v/v) Triton X-100 and 1 m*M* phenylmethylsulfonyl fluoride [35]. Proteins were separated by SDS-polyacrylamide gel electrophoresis followed by a transfer to a nitrocellulose (Schleicher & Schuell, Dassel, Germany) membrane. Monoclonal antibody against p53 was purchased from Oncogene Research Products (Ab-6, #OP43). Monoclonal anti-α-tubulin antibody (#T 9026) was purchased from Sigma. For signal detection the enhanced chemiluminescence system (Amersham, Braunschweig, Germany) was used. Scion Image Software from Scion Incorporation was used to quantify band intensities.

Statistical Analysis

An analysis of variance was performed. Significance was set to 5%. All tests were performed two-sided.

Results

In the present study, cell proliferation and sensitivity to chemotherapeutic agents of MDA-MB-435S mammary carcinoma cell transfectants expressing either wt or mutant E-cadherin molecules were compared with that of nontransfected, E-cadherin-negative parental cells. Mutant E-cadherin cDNA was cloned from diffuse-type gastric carcinomas and, as a control, wt E-cadherin cDNA was isolated from nontumorous gastric mucosa as described previously [31]. The mutations were in-frame deletions of exons 8 *(del 8)* or 9 *(del 9)* and a point mutation in exon 8 (*p8,* D370A). Expression of wt or mutant E-cadherin in MDA-MB-435S cell transfectants was demonstrated by immunoblot and immunofluorescence analysis [31]. At least two independent cell clones were investigated for each E-cadherin expression construct.

E-Cadherin Mutations Affect the Growth-Suppressive Function of wt E-Cadherin

In accordance with previous observations that E-cadherin acts as a suppressor of cell growth [5, 11], we found that the proliferation rate of MDA-MB-435S cells was significantly reduced by the expression of wt E-cadherin as compared to parental cells ($p = 0.0001$), as shown by XTT cell proliferation and viability assay (fig. 1). In contrast, E-cadherin mutations apparently interfere with the growth-suppressive function of E-cadherin (fig. 1), since *del 9*, *del* 8 and *p8* E-cadherin transfectants caused no reduction in cell proliferation.

E-Cadherin Mutations Alter the Sensitivity to Cisplatin, while Responsiveness to Etoposide and 5-FU Is Not Affected

To investigate whether wt or mutant E-cadherin molecules influence the cell death response of MDA-MB-435S cells, chemosensitivity profiles of parental, as well as wt or mutant E-cadherin-expressing cells to cisplatin, etoposide and 5-FU were compared. Exposure to cisplatin resulted in significantly reduced sensitivity of wt or mutant E-cadherin-expressing MDA-MB-435S cells as compared to parental cells in an XTT cell proliferation and a colony formation assay (fig. 2, all p values ≤ 0.001). The sensitivity profiles of parental, wt and mutant E-cadherin-expressing MDA-MB-435S cells in response to etoposide or 5-FU treatment were similar for all cell lines, as demonstrated by XTT and colony formation assay (data not shown).

p53 Expression Analysis of Parental, wt and Mutant E-Cadherin-Expressing MDA-MB-435S Cells

Next, we investigated whether the observed differences in cisplatin sensitivity were caused by differences in the p53 expression level. Immunoblot analysis performed with extracts from parental, wt or mutant E-cadherin-expressing MDA-MB-435S cells using an antibody specific for p53 revealed that the p53 expression level was similar in all tested cell lines (fig. 3). This result demonstrates that E-cadherin expression does not alter the p53

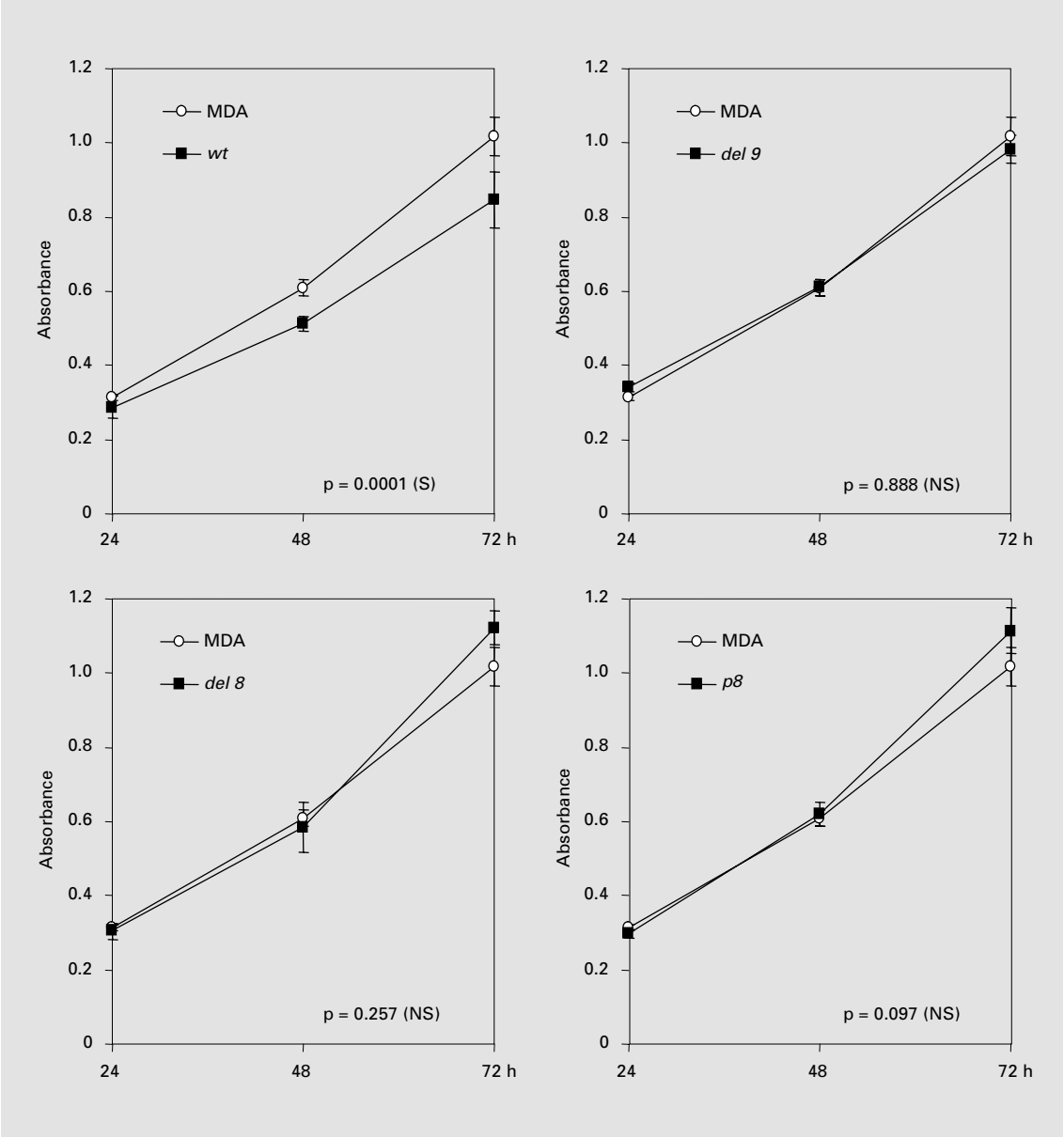

Fig. 1. Comparison of the proliferation rate of parental, wt and mutant E-cadherin-expressing MDA-MB-453S cells. Nontransfected, wt and mutant E-cadherin-expressing MDA-MB-435S cells were seeded into 96-well plates and assayed for proliferation and viability for 72 h. An increase in the number of living cells results in an increase in the activity of mitochondrial enzymes, which correlates with the amount of formazan formed by cleavage of XTT tetrazolium salt. Formazan formation was measured spectrophotometrically at 450 nm at the indicated time points. The absorbance value obtained when culture medium without cells was assayed was subtracted from the values obtained with cells. Quadruplicate determinations were performed for each time point. The bars indicate the standard deviation. One representative of three independent experiments is shown. A p value less than 0.05 was considered to be significant (S). NS = Nonsignificant.

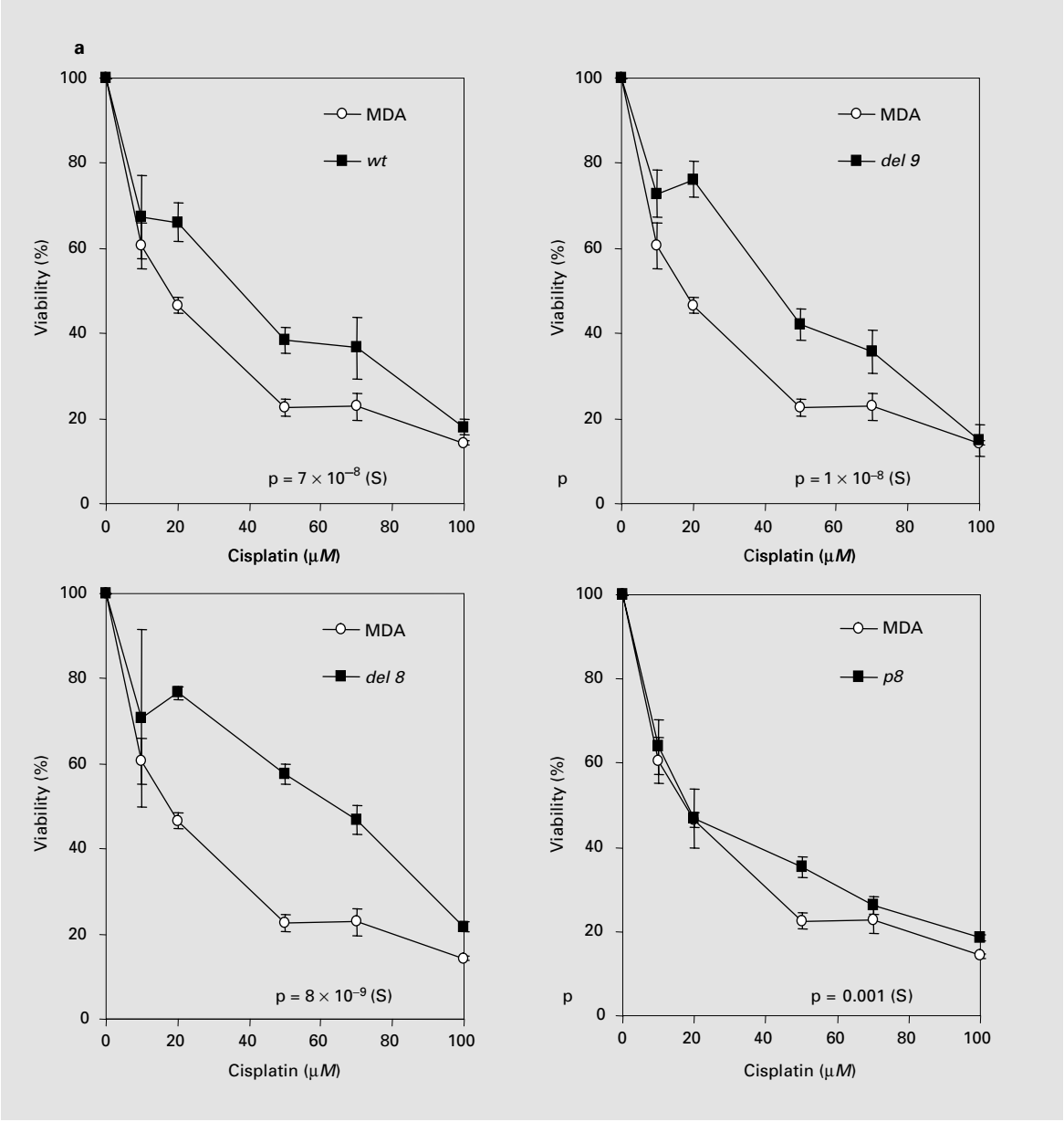

Fig. 2. Sensitivity profiles of E-cadherin-expressing MDA-MB-435S transfectants to the chemotherapeutic drug cisplatin. **a** Cells were seeded in 96-well plates and treated with increasing concentrations of cisplatin. Cell survival after a 48-hour treatment with cisplatin at the indicated doses was determined by XTT assay. The results are expressed as the percentage of absorbance values obtained with treated cells as compared to untreated cells. Quadruplicate determinations were performed for each concentration. The bars indicate the standard deviation. A p value less than 0.05 was considered to be significant.

expression level. Treatment of parental cells and transfectants with cisplatin revealed no impact of cisplatin on the p53 expression level (fig. 3). Since stable transfection of cells leads to random integration of transfected cDNA into the host chromosome and can cause genetic alterations at the integration site, the mutational hot spot region exon 5–8 of p53 was analyzed for mutations in DNA samples from MDA-MB-435S transfectants by DHPLC using a previously established protocol [34]. A change of p53 codon 266 (G266E, GGA > GAA, exon 8,

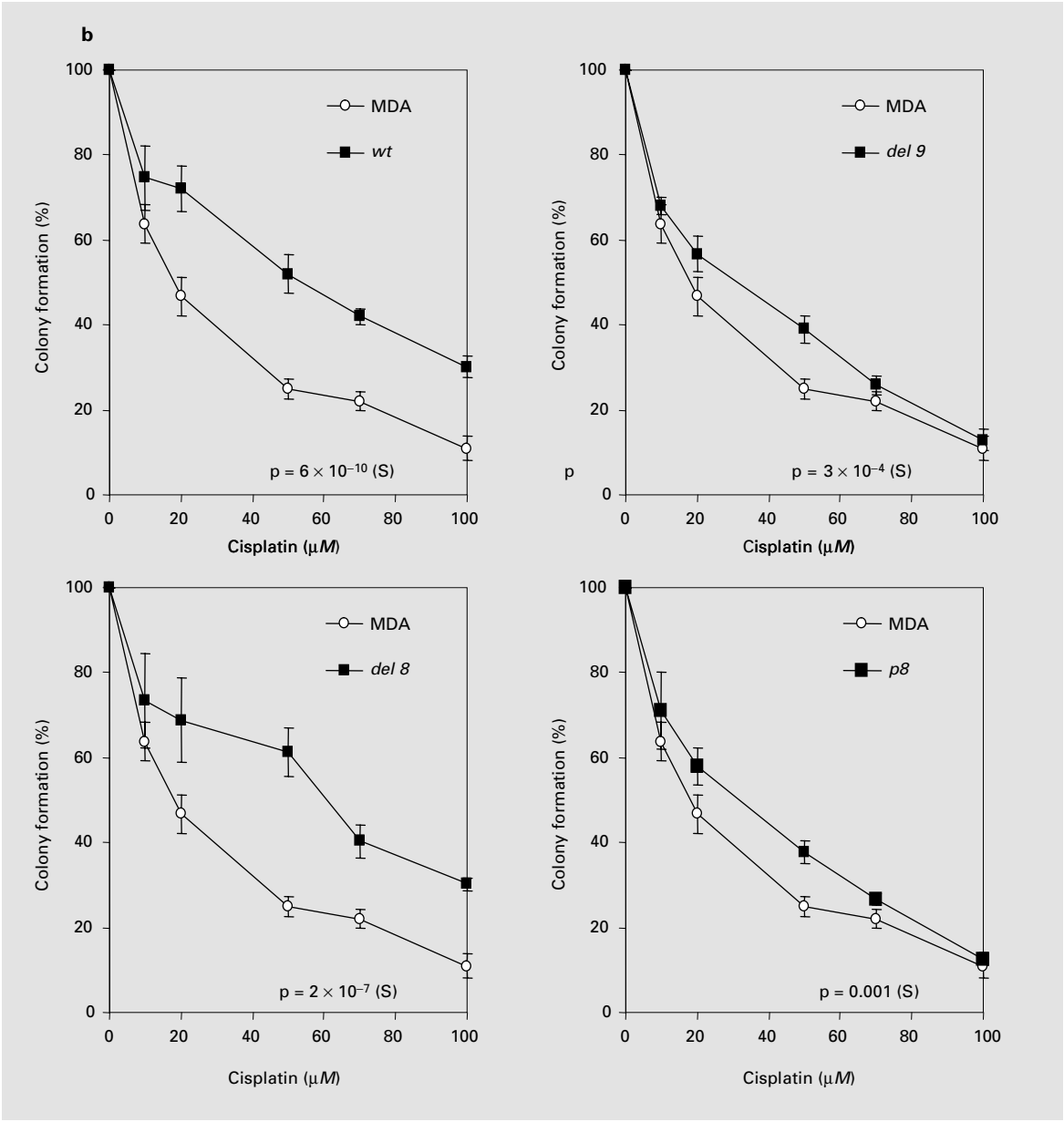

Fig. 2. b Cells were seeded in 6-well plates and treated with increasing concentrations of cisplatin. The number of colonies was determined after fixation and staining with Diff Quick reagent after 7 days. The percentage of colony formation after exposure to cisplatin was determined in relation to nontreated cells. Triplicate determinations were performed for each concentration. The bars indicate the standard deviation. A p value less than 0.05 was considered to be significant (S).

nucleotide 797) was previously described in MDA-MB-435 cells [36] and detected also in the subline MDA-MB-435S by sequence analysis [34]. The same mutation pattern was found in parental MDA-MB-435S cells, as well as in wt and mutant E-cadherin-expressing transfectants, indicating that transfection with E-cadherin cDNA did not alter the p53 mutation status. The presence of a wt as well as a mutant p53 allele indicates p53 heterozygosity (data not shown).

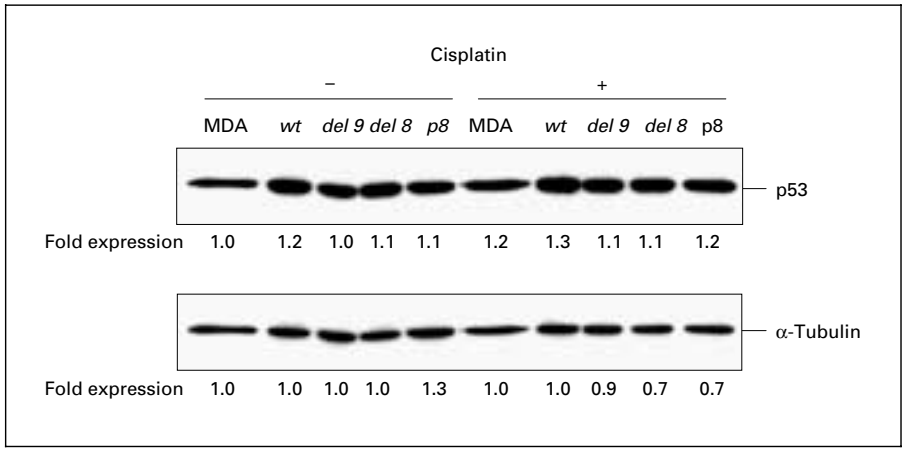

Fig. 3. p53 expression level of parental, wt and mutant E-cadherin-expressing MDA-MB-435S transfectants. The p53 expression level was analyzed by immunoblot analysis of lysates from nontransfected (MDA), *wt*, *del 9*, *del 8*, or *p8* E-cadherin-cDNA-expressing MDA-MB-435S cells using monoclonal antibody p53 (AB-6). The blot was stripped and incubated with anti-α-tubulin antibody. Equal amounts of whole cell lysates were used in each lane. Densitometric analysis of signal intensities was performed with Scion Image. p53 and α-tubulin expression is shown as fold expression in comparison to parental cells. One representative of three independent experiments is shown.

Discussion

This study was undertaken to determine the effect of wt and mutant E-cadherin on cell proliferation and drug responsiveness to cisplatin, etoposide and 5-FU. Our results suggest that the growth-suppressive function of E-cadherin was impaired by tumor-associated E-cadherin mutations. Moreover, we found that wt and mutant E-cadherin-expressing MDA-MB-435S cells were less sensitive to cisplatin treatment than E-cadherin-negative, parental MDA-MB-435S cells. In contrast, treatment of parental, wt and mutant E-cadherin-expressing MDA-MB-435S cells with etoposide and 5-FU resulted in similar sensitivity profiles for all cell lines, as demonstrated by XTT and colony formation assay.

Although the mutant E-cadherin variants investigated in this study were cloned from diffuse-type gastric carcinomas, we used MDA-MB-435S mammary carcinoma cells instead of gastric carcinoma cells as recipient cells, because MDA-MB-435S cells have been widely used for studying E-cadherin function after transfection with E-cadherin expression constructs by us and other groups [31, 32, 37, 38]. The parental MDA-MB-435S cell line lacks endogenous E-cadherin due to methylation-associated silencing of E-cadherin gene expression [39] or down-regulation of E-cadherin gene expression by snail [40]; both mechanisms have been suggested. A deletion of exon 9 of E-cadherin, which is frequently detected in diffuse-type gastric carcinoma [23], was also found in the human breast cancer cell line MPE600 [41, 42], indicating that investigation of E-cadherin mutations in breast cancer cells is of physiological relevance. However, there is recent evidence suggesting that the MDA-MB-435S cell line might not be derived from breast cancer [43, 44]. Microarray data suggested that MDA-MB-435S cells might originate from an occult melanoma [43]. Two sublines of MDA-MB-435 (MDA-MB-435S and MDA-MB-435 HGF) were subsequently analyzed by reverse transcription PCR and immunohistochemistry for the expression of a panel of genes typical of breast cells or melanocytes [44]. None of the genes characteristic of breast cancer cells were expressed in these two sublines, while several genes commonly expressed by melanocytes were detected. According to the authors, these results indicate that MDA-MB-435 might indeed be of melanoma origin.

E-Cadherin Mutations Affect the Growth-Suppressive Function of E-Cadherin

Our data suggest that mutations in the extracellular domain of E-cadherin, which affect the adhesive function of the molecule, also interfere with its growth-suppressive function. It has recently been shown that E-cadherin regulates cell growth by modulating the transcriptional activity of β-catenin [10]. Besides its role in E-cadherin-mediated cell adhesion, β-catenin forms nuclear complexes with high mobility group transcription factors [45–47]. The growth-suppressive effect of E-cadherin required the presence of its cytoplasmic β-catenin interaction domain and/or correlated strictly with the ability to negatively interfere with β-catenin transcriptional activity [10].

The mutant E-cadherin variants investigated in the present study have intact β-catenin binding sites and form complexes with β-catenin [32]. However, the E-cadherin mutations induce partially abnormal cytoplasmic and perinuclear β-catenin staining, possibly because the mutant E-cadherin variants show the same abnormal staining pattern [32]. Since β-catenin signaling regulates ex-

pression of c-*myc* [19] and cyclin D1 [48, 49], an increase in β-catenin signaling in the mutant E-cadherin-expressing MDA-MB-435S cell lines as compared to the wt E-cadherin-expressing cells could explain why the former cells have a higher proliferate rate. However, the hypothesis that mislocalization of β-catenin correlates with the transcriptional activity in our cell lines needs to be investigated. In a previous study, cell lines carrying E-cadherin mutations do not show constitutive Wnt signaling [42].

E-cadherin-dependent growth-suppression has been reported to be mediated by a mechanism which includes upregulation of the cyclin-dependent kinase inhibitor $p27^{KIP1}$ [11]. In parallel, a reduction in cyclin E-cdk2 activity and dephosphorylation of retinoblastoma protein was demonstrated. In further experiments, we will investigate the influence of E-cadherin mutations on these cell cycle-regulating proteins.

Uncontrolled expansion of tumor cells is determined by the balance between tumor cell proliferation and apoptotic cell death. E-cadherin is important for the protection of epithelial cells from apoptosis [12–14]. Therefore, E-cadherin mutations could alter the apoptotic behavior of tumor cells, thereby contributing to the expansion of tumor cells. Taken together, disregulation of cell growth in tumors with E-cadherin mutations may be advantageous to tumor cells. The underlying mechanism has to be studied in more detail.

E-Cadherin Mutations Alter Sensitivity to Cisplatin

In the present study, a difference in chemosensitivity to the anticancer drug cisplatin was detected between parental MDA-MB-435S cells and transfected cells expressing wt or mutant E-cadherin. Cisplatin sensitivity does not simply correlate with the proliferation rate. Cisplatin is a platinum-containing, DNA-damaging agent which is effective against solid tumors [50]. Cisplatin exposure leads to the formation of intrastrand cross-links. Several genes have been identified that mediate sensitivity to cisplatin [51], for instance DNA mismatch repair genes and hMSH2 and its heterodimer partners bind to cisplatin-DNA adducts. Defects in DNA mismatch repair genes produce resistance to cisplatin.

In contrast to the results obtained with cisplatin, the chemosensitivity of parental, wt and mutant E-cadherin-expressing cells to etoposide and 5-FU was similar in all cell lines, independent of the expression or mutation status of E-cadherin. A possible explanation is that these drugs act by different mechanisms: etoposide is an inhibitor of the enzyme DNA topoisomerase II which is essential for DNA replication, transcription, chromosomal segregation and DNA recombination [52]; 5-FU acts as a competitive inhibitor of thymidylate synthase and blocks both RNA and DNA synthesis [53].

Role of p53

Genetic abnormalities of the p53 tumor suppressor gene are among the most frequent mutations in tumorigenesis [54]. p53 protects cells from DNA damage by inducing either growth arrest or apoptosis in response to stress signals [55]. In response to cellular stress or DNA damage, p53 becomes activated and functional. A previous study has suggested a correlation between the p53 mutation status and growth inhibition of anticancer drugs in 60 cell lines of the National Cancer Institute [36]. In light of these findings, we investigated the genetic p53 background and p53 expression level of wt and mutant E-cadherin MDA-MB-435S transfectants. Investigation of exon 5–8 by DHPLC and Western blot analysis revealed that the p53 mutation status and expression level were unaffected by wt or mutant E-cadherin. The presence of one wt p53 allele certainly influences the chemosensitivity of MDA-MB-435S cells, since cells which are heterozygote for p53 have been shown to be more sensitive to treatment with chemotherapeutic drugs than p53-deficient cells [56].

Genetic alterations play a causative role in tumor formation and progression. Specific genetic alterations might also determine the patient's outcome after chemotherapeutic treatment. For example, it has been shown that p53 alters the chemosensitivity of cells. In the present study, we show that the presence of E-cadherin alters the sensitivity against cisplatin. Since alterations of E-cadherin found in tumors are mutational inactivation and transcriptional downregulation [21], our results may be of interest with regard to chemotherapeutic treatment of patients with abnormalities in the E-cadherin status.

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