

Loss of E-cadherin in multidrug resistant breast cancer cell line MCF-7/Adr: possible implication in the enhanced invasive ability

L. LU, D. ZHOU, X. JIANG, K. SONG*, K. LI**, W. DING

Department of General Surgery, Tenth People's Hospital of Shanghai Tongji University, Shanghai, China

*United Family Healthcare, Shanghai, China

**Department of General Surgery, First People's Hospital of Shanghai Jiaotong University, Shanghai, China

Abstract. – **BACKGROUND:** Recently, many observations have drawn researchers' attention to study drug resistance and invasion/metastasis as a single entity. However, little evidence is available about the relationship between the multiple drug resistance (MDR) and invasion so far.

AIM: To study the relationship between the multiple drug resistance and invasive-metastatic phenotypes in cancer cells.

MATERIALS AND METHODS: In this study, we compared the biological alterations of drug-resistant cells with their parental control and demonstrated that the drug-resistant cells have acquired enhanced invasive ability in addition to their acquired MDR phenotype.

RESULTS: Motility and invasiveness assays revealed that MCF-7/Adr had an invasive phenotype. Light and electron microscopy showed loss of cell-cell clustering after trypsinization, loss of tight junctions and desmosomes, ultrastructural changes to the actin network and nucleus, loss of epithelial characters, and increase in filopodia in the drug resistant cell line. A major component of cell-cell junctions, E-cadherin, was shown to be expressed on the protein and RNA levels in MCF-7 but not MCF-7/Adr cells. More protrusions (pseudopodia) on the cell surface were found in MCF-7/Adr cells, which enable the cells to migrate and invade/metastasize. Examination of ultrastructural morphology revealed that great changes occurred in drug resistant cancer cells including absence of tight junctions/desmosomes, and dislocation of cytoskeleton microfilaments. Since the cadherin/catenin complexes are further associated with the major cytoskeleton components at cell-cell junctions, we measured E-cadherin expression in drug-resistant cells. Both Western blot and immunocytochemical staining demonstrated the loss of E-cadherin expression in MCF-7/Adr cells. Further, RNA in situ hybridization results confirmed that loss of E-cadherin expression occurred at the transcriptional level.

CONCLUSIONS: The drug-resistant MCF-7/Adr cells were found to be more invasive/metastatic than their parental control, possibly related to the loss of E-cadherin expression.

Key Words:

E-cadherin, Drug resistance, Invasion/metastasis, MCF-7 cells.

Introduction

Multidrug resistance (MDR) and metastasis are two major ways cancer causes death in patients. We used the MCF-7 and MCF-7/Adr cell lines as a model to study the relationship between drug resistance and invasion/metastasis. MCF-7/Adr cells are a strain of the human MCF-7 breast adenocarcinoma cancer cell line that was selected to acquire drug resistance. MCF-7 cells were exposed to increasing concentrations of Doxorubicin, and the resulting MCF-7/Adr line was found to overexpress P-glycoprotein (P-gp), which is responsible for the drug resistance phenotype.

E-cadherin, one of the most important transmembrane cell-cell adhesion molecules, connects to the cytoskeleton through cytoplasmic catenins, and affects the adhesive, motile and morphological properties of cells. Numerous studies have shown that loss of E-cadherin function contributes to increased invasion and metastasis in carcinoma cells^{1,2}.

In this study, we compared the E-cadherin expression levels and biological alterations of the drug-resistant cells MCF-7/Adr with their parental

control, demonstrating that drug-resistant cancer cells have acquired enhanced invasive ability, which implicate a possible relationship between drug resistance and invasion/metastasis.

Materials and Methods

Cell Culture

MCF-7 and MCF-7/Adr cells were obtained from the American Type Culture Collection, Rockville, MD, USA. They were maintained in glutamine-supplemented Roswell Park Memorial Institute (RPMI) 1640 media (Gibco-BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco-BRL), 100 U/ml penicillin and 100 U/ml streptomycin, at 37°C in a 5% CO₂/95% air humidified incubator. Stock MCF-7/Adr cells, also known as NCI/ADR-RES cells³, were continuously exposed to 0.05 µM Doxorubicin for the maintenance of the MDR phenotype but were cultured in drug-free medium for at least one week before any experiment.

Transwell Migration and Invasion Assay

Cell migration and invasion assays were performed as previously described with modification^{4,5}. Briefly, for the migration assay, cells were applied to the upper well of a Boyden chamber, which were 24-transwell units with 8 µm pore polycarbonate filters (Costar; Cambridge, MA, USA). The invasion assay was identical except that the filters were pre-coated with 30 µg Matrigel (Collaborative Biomedical; Bedford, MA, USA) to simulate a basement membrane. The upper chamber was inoculated with 5 × 10⁴ cells in 250 µL of serum free RPMI 1640 medium, while the lower chamber was filled with 750 µL of medium containing 20% FBS as a chemoattractant. The Boyden chambers were incubated at 37°C in a humidified CO₂ incubator. During a three-day period, observations of motility were made on select chambers at 24, 48, and 72 h time points. Cells remaining on the upper surface were carefully removed by wiping with a cotton swab. The cells that migrated to the lower surface were stained with hematoxylin and eosin and counted under a light microscope. Five identical experiments were used per group. Values of *p* < 0.01 using Student's *t*-test were considered significant.

Immunocytochemical Staining

Both MCF-7 and MCF-7/Adr cells were grown to confluence on coverslips, and then

fixed in 95% ethanol for 30 min and rinsed twice with PBS for 10 min each. The fixed coverslips were then incubated in 3% H₂O₂ for 15 min to block the endogenous peroxidase. The cells were coated with normal goat serum for 15 min and then incubated overnight at 4°C with the first antibody (or without antibody as a negative control), with two 10 min PBS washes between each antibody application. The cells were then treated with avidin-biotin-peroxidase complex and visualized with diaminobenzidine. Nuclei were stained with dilute hematoxylin. E-cadherin antibody was purchased from Zymed (Zymed Laboratories Inc., South San Francisco, CA, USA) (Cat. No.13-5700), and the working concentration was 5 µg/ml.

Immunoblot Analysis

Total protein was extracted from each cell line by incubating cells for 30 min on ice in extraction buffer [1% Triton X-100, 50 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), 150 mM NaCl, 10 mM ethylene diamine tetracetic acid (EDTA) (pH 8.0), 10 mM Na₄P₂O₇, 100 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 µg/ml Aprotinin, and 2 µg/ml Lerpstatin]. Proteins were separated on 7.5% polyacrylamide gels and transferred to nitrocellulose filters. Equal protein loading (50 µg) was controlled by Lowry protein assay (Bio-Rad, Hercules, CA, USA). Nonspecific protein binding sites were blocked with 2% blocking reagent (Roche, Mannheim, Germany). Filters were probed with an E-cadherin monoclonal antibody (Cat. No.13-5700, Zymed, CA; 1:1500). Bound E-cadherin antibodies were detected by peroxidase-conjugated goat anti-mouse antibody followed by ECL detection (Amersham Biosciences, Piscataway, NJ, USA).

In situ Hybridization

Digoxin-labeled RNA probes were generated (Boster Biosciences, Wuhan, China) by *in vitro* transcription with oligonucleotide primers specific for E-cadherin mRNA. The oligonucleotide probe sequence against E-cadherin mRNA was: (1) 5'-TCCTG GCAGA GCCAA GAGGA GACCT GCAGC-3'; (2) 5'-TTCAT TCAGC CAGCT TCTTG AAGCG ATTGC-3'; (3) 5'-ACATG TCAGC CAGCT TCTTG AAGCG ATTGC-3'. After fixation in 4% paraformaldehyde, cells grown and fixed on coverslips were treated with 0.5% H₂O₂/methanol to block the endogenous peroxidase and digested with 3%

fresh gastric trypsin. Hybridization was carried out overnight at 37°C in hybridization buffer containing 50 ng/ml riboprobe. Post-hybridization washes included 2 × SSC (20 × SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.2) for 30 min at 37°C, 2 × SSC for 30 min at room temperature and 1 × SSC for 30 min at room temperature. Visualization of hybridization sites was performed by application of the SABC method.

Transmission Electron Microscopy

Confluent cultured breast cancer cells were scraped from the plastic culture flasks. Samples were collected after centrifugation at 500 rpm for 1 min, rinsed once with cold PBS, fixed with 2.5% glutaraldehyde/PBS for 2 hours at 4°C, and then embedded in epoxy resin. Ultrathin sections were observed with a Hitachi H-800 transmission electron microscope using an acceleration voltage of 75kV.

Scanning Electron Microscopy

Either MCF-7 or MCF-7/Adr cells were grown on coverslips placed at the bottom of six-well plates (Costar, Cambridge, MA, USA) containing growth medium. Confluent cells were rinsed with fresh serum-free medium, fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.3) for 12 hours at 4°C. The fixed cells were rinsed with

three changes of 0.1M PBS for a total of at least 2 hours, then fixed with 1% osmium tetroxide for 1.5 hours, followed by rinsing with distilled water. The coverslips were dehydrated through a graded ethanol series (10% to 100%) before critical point drying. The coverslips were dried routinely, then grounded with silver colloids and shadowed with gold. The samples were observed and photographed with a Hitachi S-570 scanning electron microscope at 20 kV (Hitachi Ltd., Tokyo, Japan).

Results

Motility Assays and Invasion Assays

MCF-7/Adr cells were significantly more migratory than MCF-7 cells in the motility assay (Figure 1a). In the invasion assay, MCF-7/Adr cells invaded the Matrigel and migrated into the lower chamber, while MCF-7 cells did not (Figure 1b).

Scanning Electron Microscopy

More cellular protrusions were seen on the surface of MCF-7/Adr cells than on the surface of MCF-7 cells (Figure 4 g and h) illustrate how this structure is vital for cancer cells to invade target tissues during metastasis.

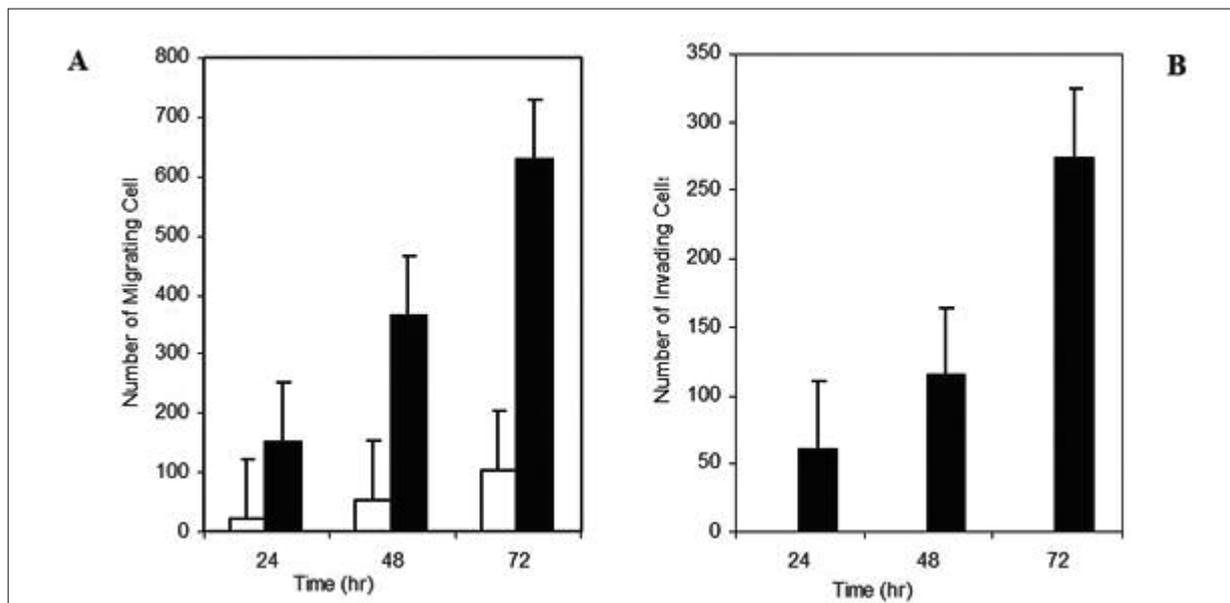


Figure 1. Migratory and invasive potential of cancer cells. *A*, Migratory ability assay: the number of cells migrating through a 8 μ M pore Boyden chamber filter counted at 24, 48, and 72 h of incubation. *B*, Invasive ability assay: the number of cells penetrating the same filters pre-coated with Matrigel and counted at 24, 48, and 72 h of incubation. MCF-7 cells: empty bars; MCF-7/Adr cells: filled bars.

Morphology of MCF-7 and MCF-7/Adr Cells

MCF-7 and MCF-7/Adr cells showed no significant difference in growth curves when cultured under normal conditions. We compared the morphology of both cell lines grown on coverslips. MCF-7 cells grew in clusters and still possessed epithelial properties such as glandular structure, while MCF-7/Adr cells grew in a disperse way, rarely demonstrated glandular structures, lost their characteristic epithelial growth pattern, and adopted a fibroblast-like cell morphology.

Furthermore, after being digested by 0.25% trypsin and suspended in medium, the two cell lines displayed different morphologies. MCF-7 cells were clustered together, while MCF-7/Adr cells appeared singly and did not associate (Figure 2 a and b).

Detection of E-Cadherin Expression

We tested for the presence of E-cadherin RNA and protein in MCF-7/Adr cells. MCF-7 cells served as a positive control known to express E-cadherin. Immunocytochemical staining for E-cadherin showed strong staining at the cell junctions in MCF-7 cells (Figure 3 a) but no staining in MCF-7/Adr cells. To be sure that the negative result was not a technical artifact, immunocytochemical staining was repeated in conjunction with hematoxylin staining (Figure 3 c and d) giving the same results. Further, Western blotting was performed (Figure 3 b) which also showed complete loss of detectable E-cadherin protein in MCF-7/Adr cells. Supporting this finding, in situ hybridization for E-cadherin RNA (Figure 3 e and f) detected RNA in MCF-7 cells only.

Transmission Electron Microscopy

Neither desmosomes nor tight junctions could be identified in MCF-7/Adr cells (Figure 4d) by transmission electron microscope (TEM), whereas they were observed in MCF-7 cells (Figure 4a). Cytoskeletal microfilaments were mainly seen adjacent to cell-cell contact regions in MCF-7 cells (Figure 4b), while they were mainly located near the nucleus in MCF-7/Adr cells (Figure 4e), consistent with loss of E-cadherin expression. Actin microfilaments observed in MCF-7 cells were long, thin, and had relatively polarized alignment compared to MCF-7/Adr cells. MCF-7 cells maintained cell polarity and secreting vesicles could be identified (Figure 4c), while MCF-7/Adr cells lost polarity. Notably, many nuclear invaginations (Figure 4f) could be seen in the drug-resistant MCF-7/Adr cancer cells.

Discussion

Previously, studies of drug resistance and invasion/metastasis have generally proceeded separately in the cancer research field. Thus, little is known about the relationship between drug resistance and cancer invasion/metastasis. More recently, observations have drawn attention to the study of drug resistance and invasion/metastasis as a single entity. The possibility of a relationship between drug resistance and invasion/metastasis phenotypes has been raised by two types of observations⁶: firstly, some drug resistant tumor cells are more invasive/metastatic relative to non-resistant parental cells; sec-

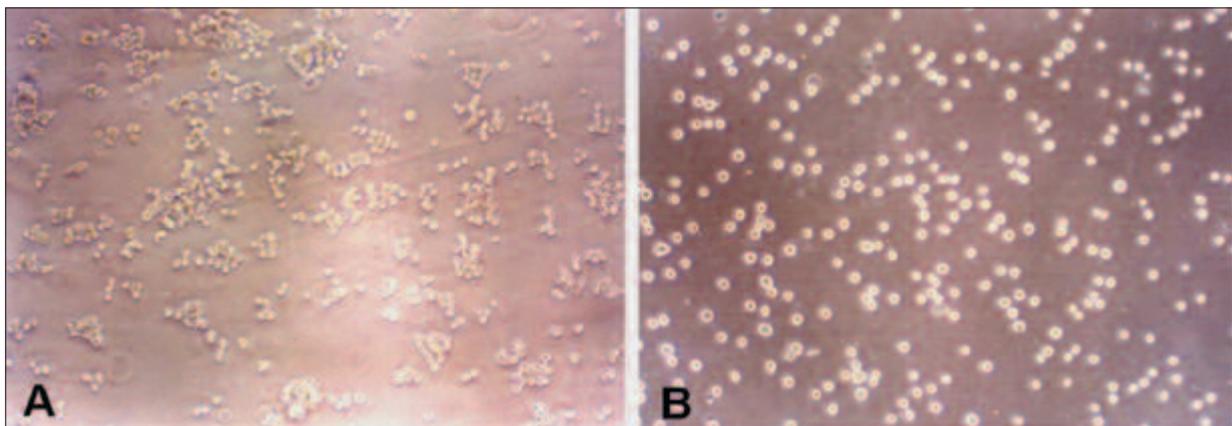


Figure 2. Completely different post-digestive state of MCF-7 cells and MCF-7/Adr cells. Phase-contrast images (100 × magnification) of cells suspended in medium after trypsinization. MCF-7 cells (A) appeared clustered, while MCF-7/Adr cells (B) appeared to be totally dispersed and dissociated.

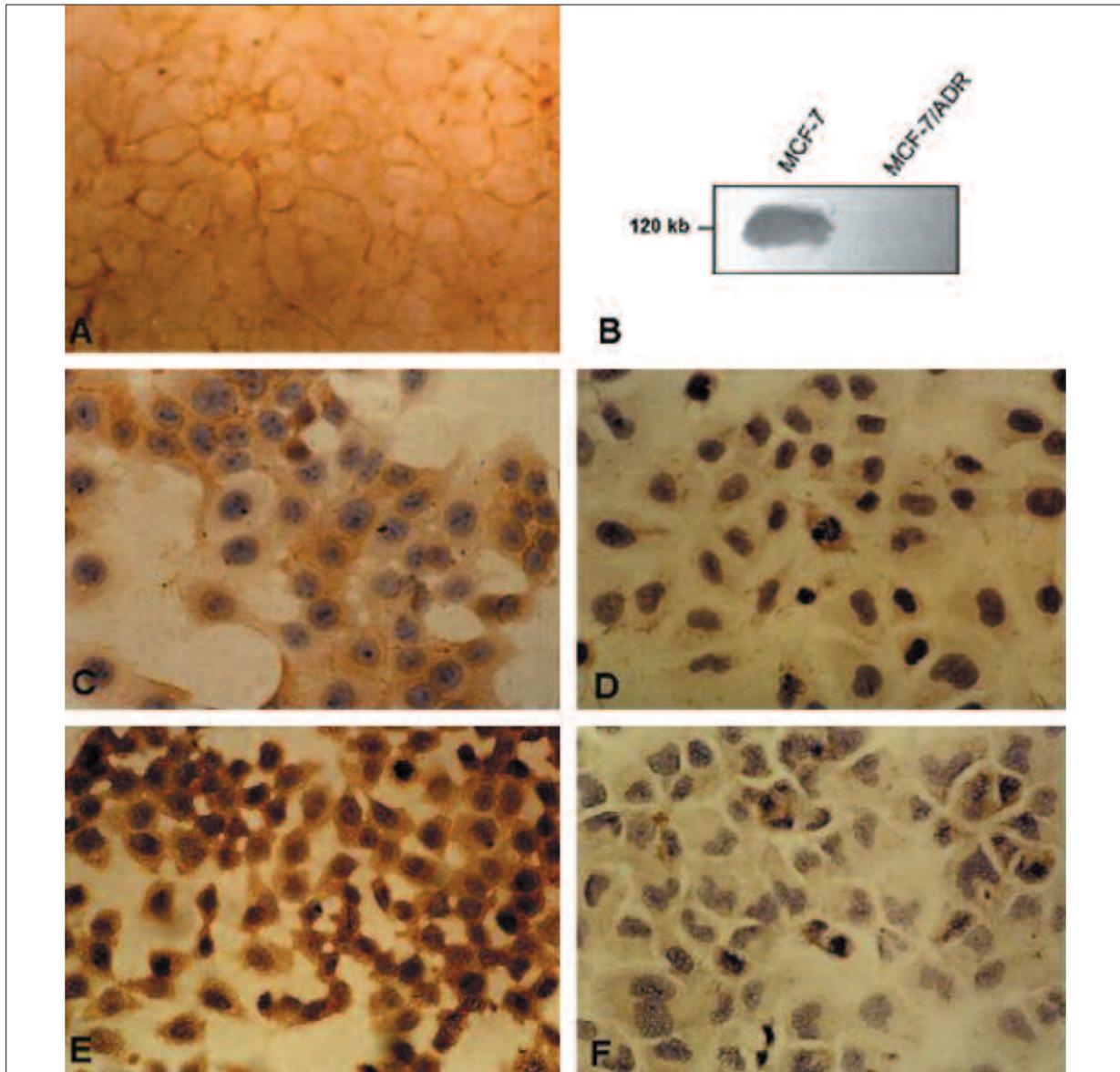


Figure 3. E-cadherin expression in MCF-7 cells and MCF-7/Adr cells. A, Immunocytochemical localization of E-cadherin in MCF-7 cells; MCF-7/Adr cells had no staining (data not shown). B, Western blot analysis with protein from MCF-7 cells (left lane) and MCF-7/Adr cells (right lane) stained for E-cadherin. Hematoxylin was used to counterstain the nuclei during immunocytochemical localization of E-cadherin in MCF-7 cells (C) and MCF-7/Adr cells (D). In situ hybridization for E-cadherin RNA expression in MCF-7 cells (E) and MCF-7/Adr cells (F).

only, in some cases, secondary (more metastatic) tumors are more resistant to chemotherapeutic drugs than their primary counterparts. In support of this, many studies have been performed. Haga et al⁷ developed a calcium-resistant human fibrosarcoma HT-1080 Cd-R cell variant, which was cross-resistant to cisplatin, and found that it was significantly more invasive than parental HT-1080 cells, as demonstrated by trans-well invasion assays *in vitro*. The

HT-1080 Cd-R cells showed increased expression of MMP-9, but not MMP-2. However, in some cases, no correlation was seen between drug resistance and cancer invasion/metastasis⁸. In this study, we compared the biology of the drug-resistant MCF-7/Adr cells with their parental control, demonstrating, as suspected, that these drug-resistant cells have acquired enhanced invasive ability in addition to their known acquired MDR phenotype.

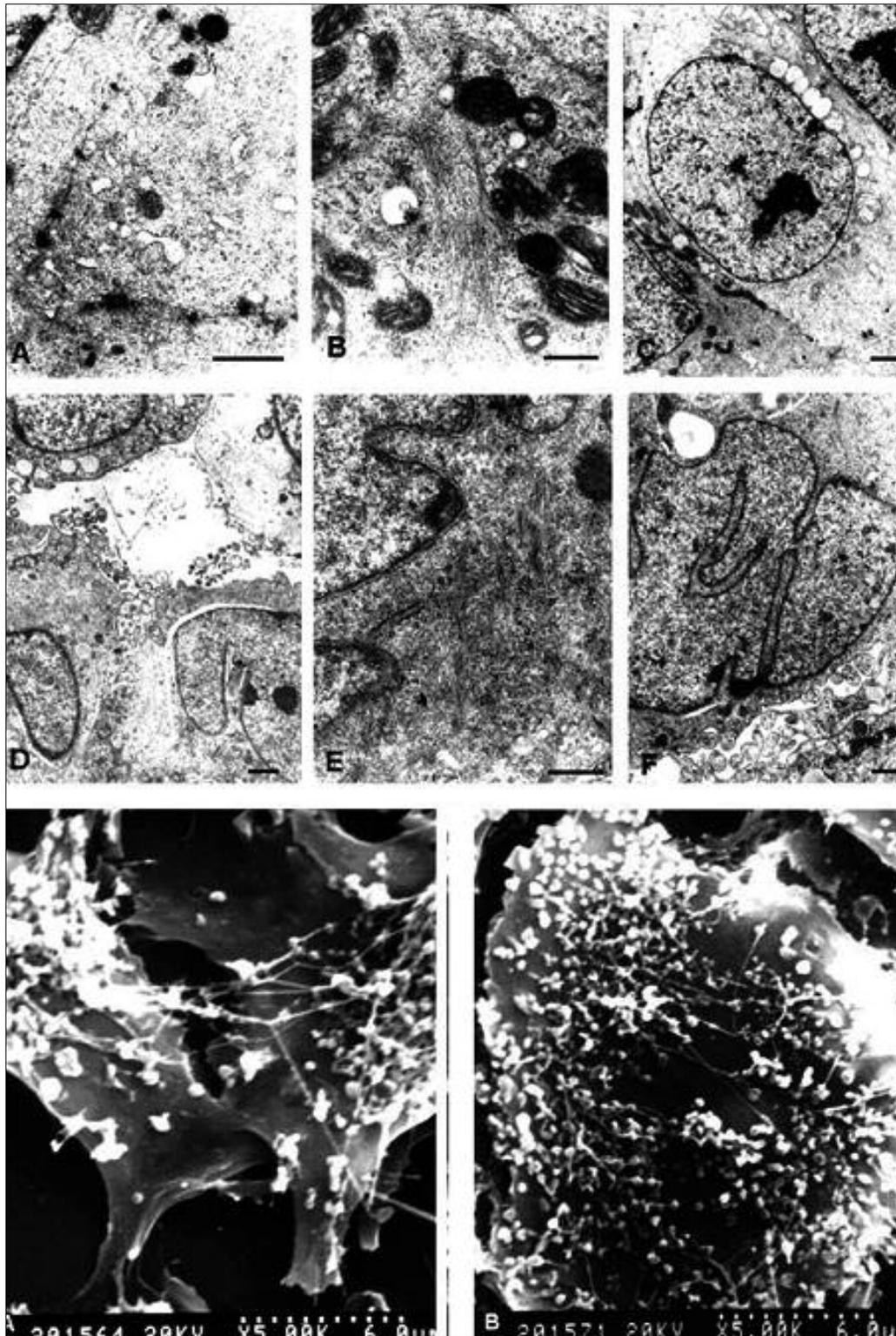


Figure 4. TEM ultrastructure related to E-cadherin/catenin complex. MCF-7 cells in panels (a), (b), (c), drug-resistant MCF-7/Adr cells in panels (d), (e), (f). (a) (d) Intercellular junctions such as tight junctions and desmosomes. (b) (e) Cytoskeletal microfilaments. Cells exhibited polarity and a regular nucleus in MCF-7 cells (c), but a depolarized cell shape and nuclear invaginations in drug resistant MCF-7/Adr cancer cells (f). Scale bars: 100 nm (a), (c), (d), (f); 50 nm (b), (e). More cellular protrusions on the surface of drug resistant cells. SEM images show MCF-7/Adr cells (h) had more protrusions on their surface than MCF-7 cells (g).

We reported the morphological changes of MCF-7/Adr cancer cells which lead to elevated invasive capability. MCF-7/Adr cells had an increased migratory and invasive potential compared to MCF-7 parental cells. Our results are consistent with previous findings by dit Faute et al who showed that MCF-7/Adr cells have increased invasive ability^{4,5,9}. It is noteworthy that the completely different morphology of these two cell lines after trypsinization (Figure 2a and b) originally attracted us to explore why MCF-7/Adr cells became more invasive. The pipetting force used to suspend the cells in medium is similar to a shearing stress. In response to low shearing stress, E-cadherin negative tumor cells are more likely to disaggregate than E-cadherin positive cells. Moreover, cell adhesion molecules play important roles in cells maintaining morphology, and down-regulation of E-cadherin correlates with cellular dedifferentiation and glandular disintegration¹⁰⁻¹⁵. It's reported that E-cadherin plays a central role in the maintenance of cell polarity and its loss during tumorigenesis is associated with poorly differentiated cancers and a poor prognosis. Based on the morphology of both cell lines, we hypothesized that alterations in E-cadherin expression occurred in the MCF-7/Adr cells as MCF-7 is known to be E-cadherin positive. Immunocytochemical staining and Western blot analysis using anti-E-cadherin antibodies demonstrated that loss of expression occurred in the MCF-7/Adr cells. Combined with the in situ hybridization data and Northern blot analysis reported elsewhere^{10,16}, we concluded that the loss of E-cadherin expression occurred at the transcriptional level. Furthermore, ultrastructural changes correlated with E-cadherin expression status^{10,16} were observed using TEM. Loss of E-cadherin expression resulted in the loss of intercellular junctions between MCF-7/Adr cells. From the clinical point of view, down-regulation of E-cadherin expression correlates with a strong invasive potential, resulting in poor prognosis in human carcinomas¹⁷. E-cadherin is connected to actin by catenin, then the cadherin/catenin complexes further associate with the major cytoskeletal components at cell-cell junctions. Loss of E-cadherin leads to disintegration of the E-cadherin/catenin complex. Actin microfilaments, which are the major component of the cytoskeleton and driving the cell motility¹⁸, were observed to have become disorganized in MCF-7/Adr cells accordingly. Remodelling of the actin cytoskeleton and E-cadherin cleavage is contributing to increased migratory and invasive properties¹⁹.

The loss of E-cadherin expression in the MCF-7/Adr cells, resulting from the down-regulation of E-cadherin expression at the transcriptional level, contributes to enhanced cancer cell motility and invasiveness^{1,3,20-24}. In addition, we observed ultrastructural changes on the cell surface using scanning electron microscope (SEM). There were more cellular protrusions or filopodia on the MCF-7/Adr cells than the MCF-7 cells, such as invadopodia, a kind of specialized actin polymerization-driven protrusions used by invasive carcinoma cells²⁵. As illustrated in Figure 5c, the cellular protrusions are the morphological basis for cancer invasion into the target tissue. Taken together, the loss of E-cadherin expression in the drug-resistant cancer cells and the distinctive ultrastructural changes account for the elevated invasive capability of MCF-7/Adr cells. The morphological presence of more cellular protrusions on the surface of the drug-resistant cells is the most important factor relating to elevated invasive capability²⁶.

Chemotherapy fails in cancer patients either because of the initial presence of or subsequent mutation and selection for drug resistant cancer cell clones²⁷. It is rare that chemotherapy results in cure of cancer, although complete or partial remission may occur. Cells displaying the MDR phenotype are cross-resistant to a wide range of structurally unrelated drugs. The underlying mechanisms²⁸ include overexpression of the multidrug resistance protein P-gp (P-glycoprotein) which acts as a pump to decrease the effective intracellular cytotoxic concentration²⁹. One proposed approach to treat drug-resistant cancer cells is to re-sensitize the cells to chemotherapies, allowing drugs to reach effective intracellular concentrations. However, the results of the clinical trials have been disappointing. It is assumed that the decreased tumor responsiveness to chemotherapy results from decreased drug accumulation due to MDR. However, Yang et al^{30,31} reported that treatment of animals bearing *MDR1*-transfected leukemic cells with P-gp substrates (i.e., drugs that are transported by P-gp) significantly worsened host survival compared to treatment with vehicle or non-P-gp substrates. They found that drug-induced acceleration of cancer was associated with increased invasion/metastasis. Likewise, Weinstein et al³² reported an association in colon carcinomas between *MDR-1*/P-gp expression and enhancement of local tumor aggressiveness. In all but one of the 95 clinical specimens of primary colon ade-

nocarcinomas, MDR-1/P-gp positive invasive carcinoma cells were present at the leading edge of the tumor. There was a significantly greater incidence of vessel invasion and lymph node metastases in MDR-1/P-gp positive cases, indicating that MDR-1/P-gp positive invasive colon cancer cells may have an increased potential for dissemination.

Several mechanisms have been proposed to explain the increased invasiveness of MCF-7/Adr cells, including increased vimentin expression following loss of estrogen receptor expression and the central role of sphingolipid signaling in apoptotic mechanisms and chemoresistance in estrogen receptor-positive breast cancer³³. But we correlated loss of MCF-7/Adr E-cadherin transcription with phenotypic changes in morphology and invasive behavior. Generally, E-cadherin and catenins stain more strongly in well-differentiated cancers than in poorly differentiated tumors, which maintain their cell adhesiveness and are less invasive. Any alteration in E-cadherin and catenin expression or the absence of these proteins from cell membranes has also been detected in pre-invasive lesions³⁴. Similarly, down-regulation of E-cadherin has been observed in poorly differentiated tumors and in highly invasive tumor cell lines, suggesting that the cell adhesion promoted by this molecule may be important for the maintenance of an epithelial phenotype and for the suppression of tumor invasion^{1,2,35}. In addition, the disconnection of β -catenin from the E-cadherin/catenin complex activates Wnt signaling, which aggravates the malignant transformation of cells. Thus, we conclude that E-cadherin silencing might play very important roles in metastatic transformation.

In summary, tumor cells that survive chemotherapy treatments often recover with increased resistance to the agents used in the original treatment program as well as to other related drugs. Such resistance in surviving cancer cells may develop by one or more mechanisms including elevated invasive capability which accelerates the disease progress. The simultaneous occurrence of elevated invasive capability, loss of E-cadherin expression, and acquired MDR in the same cells demonstrates the strong links between drug resistance and tumor invasion³⁶. A clear understanding of how cell-cell adhesion is linked with the multidrug resistance should aid in the development of novel and effective anticancer strategies.

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