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Update on the molecular profile of the MDA-MB-453 cell line as a model for apocrine breast carcinoma studies

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Abstract. Apocrine carcinoma of the breast has recently been refined through gene expression profiling. Due to various pathological studies, we compared the results with the MDA-MB-453 breast cancer cell line, a proposed model for apocrine breast carcinoma. The MDA-MB-453 cell line is androgen receptor-positive and 'triple-negative' in respect to estrogen receptor-α, progesterone receptor and the Her-2/neu protein expression. Cytogenetic analysis of the cell line revealed a hypertriploid clone characterized by extensive numerical and structural abnormalities including loss of the 9p.21 locus (*P16-INK4a* gene), also evidenced by the lack of $p16^{INK4A}$ protein expression in Western blot analysis and immunocytochemistry assays. Gains of chromosomes 7 and 17 without underlying *EGFR*, *HER-2/neu*, and *TOP2A* gene amplification were also observed. A mutation in the *K-RAS* gene (Gly13Asp GGC>GAC) was identified in the cell line, which was not observed in the six patient samples of apocrine breast carcinomas examined. Similarly, constitutive activation of the MAPK/ERK signaling pathway and deregulation of cell cycle proteins (p16-/pRb-/cyclin D1+ phenotype) with exceedingly high proliferation observed in the MDA-MB-453 cell line were not found in the tissue samples. In conclusion, the MDA-MB-453 cell line shares certain features with apocrine breast carcinoma but differs from patient tissues with regard to various significant characteristics, limiting the value of this cell line as a model for human apocrine breast carcinoma investigations. In contrast to the cell line, EGFR-positive apocrine carcinomas do not harbor *K-RAS* gene mutations, rendering these tumors amenable to targeted therapy with EGFR inhibitors.

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Introduction

Breast cancer is the most commonly occurring malignancy in women worldwide. Various histological and molecular subtypes of breast cancer have been identified with different biologic implications (1-3). Apocrine differentiation (metaplasia) is frequently observed in the mammary epithelium; however, invasive apocrine carcinomas are rare, constituting less than 5% of all breast carcinomas (1,4). Apocrine lesions of the breast are characterized by over-expression of the androgen receptor (AR) along with loss of estrogen receptor-α (ER-α) and the progesterone receptor (PR) (5-7). Molecular studies of invasive apocrine breast carcinoma have shown a specific molecular apocrine profile based on the AR expression that divides ER-negative breast carcinomas into two different clusters: ER-/AR- (basal) and ER-/AR+ (molecular apocrine cluster) (8).

Breast cancer cell lines are widely used for experimental research as models of various subtypes of breast carcinomas (9-12). The MDA-MB-453 breast cancer cell line, obtained from a malignant pleural effusion of a 48-year-old female, has been suggested as a model for the molecular apocrine breast subtype (13-15). The cell line exhibits a characteristic apocrine carcinoma steroid receptor profile: ER-α-negative, PR-negative, and AR-positive (8,13). Increased proliferation in response to androgens is the key feature of the cell line, which can be blocked by anti-androgens, such as flutamide (13,16,17). Her-2/neu activity has been well documented in this cell line, as well as the existence of a functional cross-talk between AR and Her-2/neu, involving the MAPK/ERK1/2 pathway (13,14). These features also tend to characterize a substantial proportion of invasive apocrine carcinomas of the breast (18-20).

The aim of the present study was to further characterize the MDA-MB-453 cell line and to correlate it with the results obtained from the apocrine breast carcinoma samples of patients.

Materials and methods

Breast cancer cell lines and treatment. Human breast cancer cell line MDA-MB-453 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and used for analysis and experiments, while MDA-MB-231, MCF-7

Table I. List of the antibodies used in the study.

Antibody	Manufacturer
ER-α (clone 6F11), PR (clone 16), Her-2/neu (Clone CB11), cyclin D1	Ventana Medical Systems
EGFR, MAPK1/2 (ERK1/2), pERK1/2 (Thr202/Tyr204)	Cell Signaling Technology
p16 (mouse monoclonal IgG1κ)	Cell Marque
Cyclin D1 (clone SP4 rabbit IgG), GCDFP-15 (clone 23A3 mouse IgG2A κ), ER-α (Cat no. RB-9016-P)	Neomarkers
EGFR (PharmDX diagnostic kit), Topoisomerase-IIα (Clone Ki-S1), Cytokeratin 5/6 (D5/16B4), p63 (clone 4A4)	DakoCytomation
Rb (IF8): sc-102, pRb(Ser795), p16 (N-20: sc-467), p53 (DO-1): sc-126, Actin [(I-19): sc-1616], AR (clone AR441: sc-7305), pEGFR (Tyr845), Her-2 (Neu (CB11): sc-52349), p-Neu (Tyr 1248)-R: sc-12352-R	Santa Cruz Biotechnology

and BT-474 cell lines from the ATCC served as controls. MDA-MB-453, MDA-MB-231 and BT-474 were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) at 37˚C in a humidified CO_2 incubator. The MCF-7 cell line was cultured in Improved modified Eagle's medium (IMEM, Invitrogen) supplemented with 10% FBS. Prior to the experiments, the cells were cultured in phenol-red-free DMEM (Invitrogen) for 24 h, and then in serum-free DMEM overnight. U0126 (MEK1/2 inhibitor, Cell Signaling Technology, Danvers, MA, USA), a highly selective inhibitor of MEK1/2, was used.

Breast tissue samples. Formalin-fixed paraffin-embedded blocks of 8 cases of apocrine carcinoma (seven invasive and one *in situ* case) that corresponded to the cell line profile (ER-α-, PR-, AR+ and Her-2/neu -/+) were selected from the previously well-characterized cohort (18) and used for comparative analysis.

Protein expression analysis. Protein expression was analyzed by Western blot analysis in cell lysates or by immunocytochemistry (ICC) and immunohistochemistry (IHC) on formalin-fixed and paraffin-embedded cell blocks prepared from the MDA-MB-453 cell line and tissue blocks from breast tissue samples (the list of antibodies is shown in Table I). For ICC and IHC analysis, commercially available detection kits and automated staining procedures were employed (18). Cell lysates, immunoprecipitations and Western blot analysis were carried out using standard procedures as previously described (21,22). Signals were detected by enhanced chemiluminiscence (ECL; Amersham, GE Healthcare Biosciences, Pittsburgh, PA, USA).

A positive $p16^{INK4A}$ expression was defined as the presence of nuclear and/or cytoplasmic staining, and the percentage of positive cells was recorded (23,24). For ER-α, PR, AR, cyclin D1, p53, p63 and topoisomerase-IIα expression, only nuclear labeling was scored (24,25). For Her-2/neu and EGFR proteins, only membranous staining was considered positive. The scoring was carried out according to the manufacturer's (EGFR, Dako, Carpinteria, CA, USA) and American Society of Clinical Oncology/College of American Pathologists' guideline recommendations (Her-2/neu protein) (26). GCDFP-15 and CK5/6 proteins were considered positive if any membranous/cytoplasmic staining was observed.

Conventional cytogenetics. G-banding procedures were performed on metaphase cells. Metaphase chromosomes were banded with Wright trypsin and karyotypes were described following the established international guidelines (27).

Fluorescent in situ hybridization (FISH). FISH was performed to evaluate copy numbers at *EGFR*, *TOP2A* and *HER-2/neu* loci. Chromosome enumeration probes CEP7 and CEP17 were used as indicators of chromosome copy numbers (Abbott Molecular Inc., Des Plaines, IL, USA). A total of 100 nuclei were scored per sample. A ratio of *HER-2/CEP17* >2.2 was defined as gene amplification; a ratio of 1.8-2.1 was interpreted as borderline, and a ratio of <1.8 was defined as negative. The same criteria were used for the interpretation of EGFR/CEP7 and TOP2A/CEP17 ratios, respectively. For statistical purposes, equivocal FISH results (ratio of 1.8-2.1) were considered negative (27). Polysomy 7 and 17 were defined as ≥3 *CEP* signals per cell (18,28).

Flow cytometry. Flow cytometry (FC) was applied to measure the S-phase fraction of MDA-MB-453 cells. Cells at ~50% confluence were harvested and 1 ml cold 70% ethanol was slowly added to the cell pellet while vortexing. Ethanol-fixed cells were treated with 100 μg/ml RNaseA and 50 μg/ml

Table II. A summary of the key findings in the cell line and apocrine carcinoma tissue samples.

Feature	MDA-MB-453	Apocrine carcinoma tissue samples
Estrogen/progesterone receptor	Negative	Negative[b]
Androgen receptor	Positive[a]	Positive[b]
GCDFP-15 protein	Negative	Positive[c]
HER-2/neu status	Low protein expression[a]/no gene amplification	High protein expression common/gene amplification[b]
EGFR status	Low protein expression[a]/no gene amplification	High protein expression common/no gene amplification[b]
KRAS gene status	Mutated (Gly13Asp GGC>GAC)	Not mutated
BRAF gene status	Not mutated	Not mutated
$p16^{INK4A}$ protein	Lost due to gene deletion at 9p.21/no protein detected	Present (variable cytoplasmic/nuclear staining)
Cyclin D1 protein	Highly over-expressed	Low expression
Polysomy 7 and 17 (CEP7 and 17)	Present	Frequently observed[b]
Basal markers (CK5/6, p63)	Low expression	Low expression[d]

[a]By Western blot analysis; [b]Vranic *et al* (18); [c]Tavassoli et al (1); [d]Weigelt *et al* (4).

propidium iodide (PI) in PBS at room temperature for 30 min. Flow cytometry of cell cycle distribution was performed using a FACSCalibur flow cytometer (BD-Biosciences, San Jose, CA, USA). The results were analyzed using Multicycle for Windows.

Gene mutation analysis. These assays included the detection of 12 mutations in codons 12 and 13 of the *K-RAS* gene as well as the V600E mutation in exon 15 of the *B-RAF* gene. The analysis was performed using a Mutector II assay, using proprietary Shift Termination Assay (STA) technology (TrimGen Corporation, Sparks, MD, USA) following the previously described protocol (29). For quality control of the *K-RAS* mutation analysis, we used a colon cancer biopsy known to be positive for *K-RAS* mutation, while a case of malignant melanoma served as a positive control for *B-RAF* mutation.

Statistical analysis. Quantitative data of the experimental studies were expressed as the mean ± SD. The student's t-test was used to test the differences between responses in the exposed and control groups. Statistical Package for the Social Sciences version 17.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. $P<0.05$ was considered to be statistically significant.

Results

Profiling of MDA-MB-453 cell line. A summary of the key findings and comparison with the apocrine carcinoma tissue samples is shown in Table II. The MDA-MB-453 cell line was negative for ER-α, PR and Her-2/neu protein on ICC, whereas AR was found to be positive by Western blot analysis. Notably, the ICC assay demonstrated no nuclear staining but predominantly cytoplasmic distribution of AR, whereas the GCDFP-15 protein was absent. Western blot analysis revealed low levels of Her-2/neu and phosphorylation of the Her-2/neu protein at Tyr1248 in comparison with BT-474 cells, an ER-positive breast cancer cell line with *HER-2/neu* gene amplification. Western blot analysis revealed that levels of total EGFR and phosphorylation of EGFR at Tyr845 were lower in MDA-MB-453 cells compared with EGFR-over-expressing MDA-MB-231 cells. In contrast, the ICC assay revealed a strong EGFR protein expression (score 3+). FISH analysis revealed neither *HER-2/neu* (*HER2/CEP17* was 3.96/3.68, ratio: 1.08) nor *EGFR* gene amplification (EGFR/CEP7 was 3.23/2.90, ratio: 1.11). Gains of chromosomes 7 and 17 (CEP7 and 17) observed with FISH were further confirmed using conventional cytogenetic analysis, which revealed a hypertriploid clone characterized by extensive numerical and structural abnormalities including gains of chromosomes 7, 11, 17, 19 and 21 and losses of chromosomes X, 3, 4, 9, 13, 14, 16 and 18.

Of particular functional importance was the deletion of the 9p21 locus that harbors the *INK4A* gene ($p16^{INK4A}$) and *INK4B* gene (p15), as evidenced by the loss of the $p16^{INK4A}$ protein expression in Western blot or ICC analysis (Fig. 1A).

CK5/6 was completely absent, whereas the p63 protein expression with defined nuclear staining was observed in approximately 10% of the cells.

MDA-MB-453 cells exhibit a high proliferation rate. MDA-MB-453 cells exhibited an excessive proliferation rate under optimal culture conditions as its S-phase fraction measured up to 70% by flow cytometry. This rate was consistent with the cyclin D1 over-expression (positive in ~70% of the cells), inactivation of retinoblastoma protein

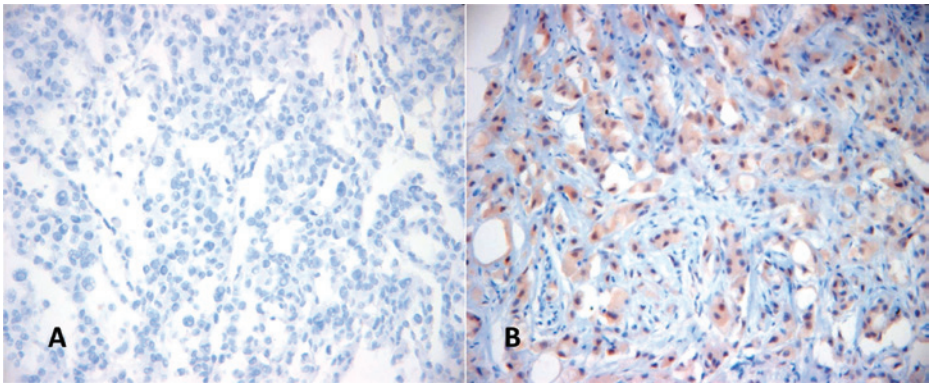

Figure 1. p16^{INK4A} protein was completely absent in MDA-MB-453 cells (A) whereas apocrine carcinoma tissue samples predominantly retained a membranous and cytoplasmic pattern of staining (B).

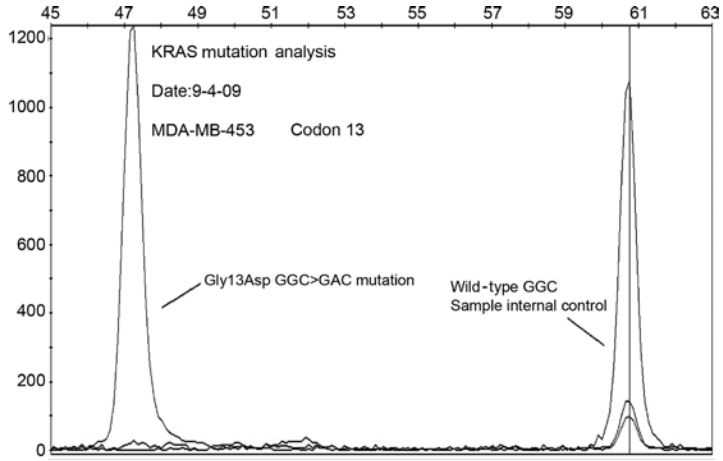

Figure 2. Mutational analysis of the *K-RAS* gene revealed codon 13 mutation (Gly 13 Asp GGC>GAC), a mutation that constitutively activates K-Ras in the MDA-MB-453 cell line.

(Rb), measured by its phosphorylation at Ser795, and the loss of p16^{INK4A} protein expression. The cell line also exhibited a high p53 protein expression measured by both Western blot and ICC analysis. Consequently, topoisomerase-IIα was also strongly expressed in approximately 70% of the cells (without underlying *TOP2A* gene amplification, *TOP2A*/CEP17: 2.86/2.80, ratio: 1.02). When cultured in a serum-free medium for 24 h, the cell line remained highly proliferative with an S-phase fraction of approximately 40%. The cell line was also capable of sustained proliferation in a serum-free medium for up to 48 h, as demonstrated by the S-phase fraction.

MDA-MB-453 cells harbor a K-RAS mutation and exhibit constitutive activation of the MAPK/ERK pathway. Mutational analysis of the *K-RAS* gene revealed codon 13 mutation (Gly 13 Asp GGC>GAC), a mutation that constitutively activates K-Ras in the MDA-MB-453 cell line (Fig. 2), whereas there is no gene alteration in the *B-RAF* gene. Western blot analysis confirmed that ERK1/2 is highly phosphorylated in cells irrespective of the concentration of the fetal calf serum, and culture conditions. In addition, MDA-MB-453 retained high ERK activity even after 48 h in serum-free medium, and was highly sensitive to the specific MEK1/2 inhibitor U0126. Low concentrations of U0126 (0.1 μM) led to a partial inhibition of ERK1/2 phosphorylation after 12 h. The exposure of MDA-MB-453 cells to U0126 at a concentration of 10 μM for various time periods (from 15 min to 24 h) led to a markedly reduced S-phase fraction as measured by flow cytometry (Fig. 3A and B). Thus, 12 h treatment of the MDA-MB-453 cells with U0126 resulted in a significantly reduced proliferation rate in the experimental group in comparison with the control group (22.6 vs. 38.3%, reduction ~42%, $p<0.001$, Student's t-test). Western blot analysis with phospho-specific anti-ERK1/2 antibody also showed that U0126 abolished ERK1/2 phosphorylation in MDA-MB-453 cells.

Breast tissue sample analysis. Of 5 tested patient samples of the apocrine carcinomas, 1 exhibited an absence of p16^{INK4A} protein expression, 3 samples (two invasive and one *in situ* carcinoma) had predominantly cytoplasmic p16^{INK4A} expression (observed in 20-80% of the tumor cells), whereas only 1 case retained a nuclear p16^{INK4A} expression intensity, compared with the positive control (Fig. 1B). Cyclin D1 expression was observed in 3 out of 5 cases. However, only 2 of these cases reached more than 10% of the positive cells with predominantly weak to moderate nuclear intensity.

K-RAS and *B-RAF* mutational analysis performed on 6 invasive apocrine carcinoma samples showed no gene altera-

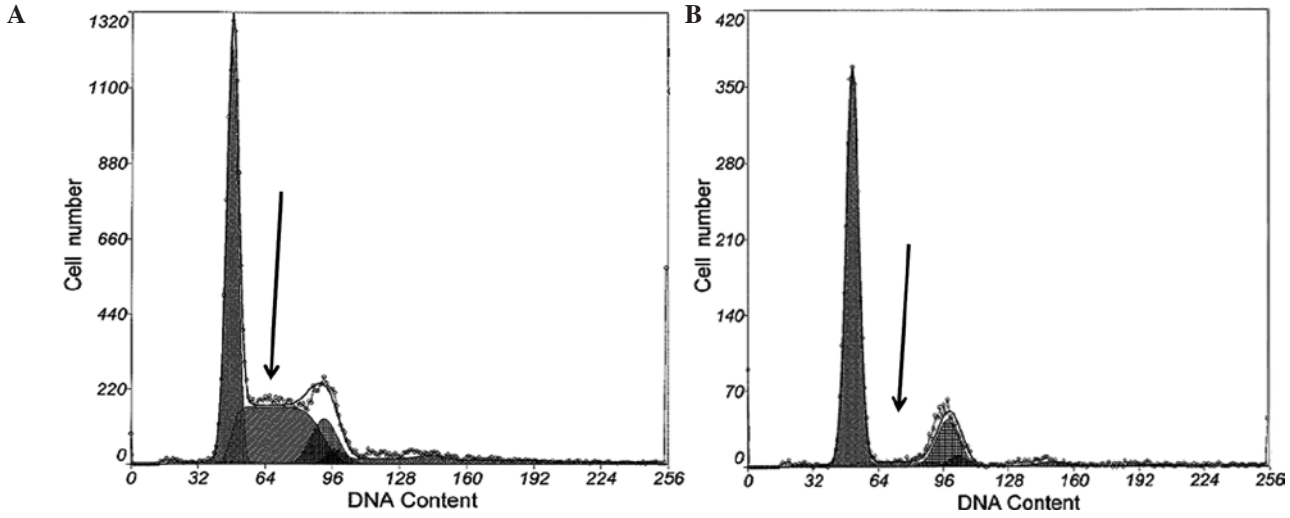

Figure 3. The exposure of MDA-MB-453 cells to U0126 at a concentration of 10 μM at different time periods (from 15 min to 24 h) led to a dramatically reduced S-phase fraction in the cells as measured by flow cytometry. Black arrows indicate S-phase fraction after 24 h in control group (A) where S-phase was ~39%, and exposed group (B) where S-phase was ~6%.

tions in any of the studied cases. Notably, these cases had a strong *EGFR* protein over-expression on IHC (scores 2+/3+ on IHC) without underlying EGFR gene amplification (18).

Discussion

The present study describes complex and multiple cytogenetic and molecular alterations in the MDA-MB-453 cell line that is currently considered to be a model for breast apocrine carcinoma. Conventional cytogenetic analysis revealed that MDA-MB-453 is a hypertriploid cell line that corresponds closely to its metastatic origin and high malignant potential (10). We also observed considerable discrepancies between published cytogenetic findings regarding the cell line and our own results, although certain previously reported cytogenetic findings, including gains of chromosomes 7 and 17 and loss of the 9p.21 locus (*CDKN2* gene) (30), are also observed in our study. Thus, previous studies (31,32) reported *HER-2/neu* gene amplification in the MDA-MB-453 cell line, whereas our analysis did not support those findings. FISH and conventional cytogenetic analysis revealed a gain (polysomy) of CEP17 in only a small population of cells (~5% of the counted cells) that carried *HER-2/neu* gene amplification. Levels of total Her-2/neu expression and phosphorylation of Her-2/neu at Tyr1248, often associated with *HER-2/neu* gene amplification (33), were lower in comparison with the positive cell control (BT-474 cell line). Belsches-Jablonski *et al* (34) also revealed a low Her-2/neu protein expression in the MDA-MB-453 cell line, whereas Kao *et al* (11) found low copy numbers of the *HER-2/neu* gene in the cell line using a quantitative polymerase chain reaction, and thus classified MDA-MB-453 cells as an HER-2-negative cell line. Consequently, the classification of the cell line varies from luminal (12,31,32) or 'weakly luminal' (10) to an apocrine cell line model (8,13-15). We propose that the observed disparities are caused by the prolonged cell culture, which may modify genetic/phenotypic properties of the cell line (9,10), and further stress the importance of a laboratory self validation of the cell line.

Our study reports for the first time a codon 13 mutation in the *K-RAS* gene followed by constitutive MAPK/ERK activation in the MDA-MB-453 cell line (35,36). The *K-RAS* gene is an integral part of the MAPK signaling pathway, whose activation strongly correlates with the degree of the *K-RAS* gene activation (36). The MAPK/ERK signaling pathway is crucial in the regulation of cell proliferation, differentiation, survival and metastasis through ultrasensitive switch-like responses to various stimuli depending on the strength and duration of stimulation (35,37-40). Some of these effects, such as prolife-ration, may be abolished by the targeted inhibition, as we demonstrated using the specific MEK1/2 inhibitor U0126 in our study.

K-RAS gene mutations (usually restricted to codons 12, 13 and 61) are frequently observed in human tumors (~15%) (41) and established breast cancer cell lines (35). However, Hollestelle *et al* (35) reported *PTEN* but not *K-RAS* gene mutation in this cell line, although these authors found that 13% of the studied breast cancer cell lines harbored *K-RAS* gene mutations. Furthermore, results obtained from the cell line were not in concordance with the data from the breast cancer samples, which is consistent with findings of previous reports that have confirmed the lack of *K-RAS* mutations in breast carcinomas (36,42-45). Notably, the lack of *K-RAS* mutations in apocrine carcinomas may become a potential therapeutic benefit, since the tested cases were EGFR-positive and thus are potentially amenable to targeted anti-EGFR therapy (44). *B-RAF* gene mutations, mainly involving the V600E locus, have also been described in a wide range of human tumors with the highest frequency in malignant melanoma (41). These mutations are rarely observed in breast carcinoma (41). Neither the cell line nor apocrine breast carcinoma samples harbored the *B-RAF* gene mutations.

Another significant finding in this study was the deletion of the 9p21-22 region, responsible for the complete loss of the $p16^{INK4A}$ protein as confirmed by ICC and Western blot analysis. This finding is consistent with a recent study that reported an allelic loss at 9p21 loci in MDA-MB-453 cells with a barely detectable $p16^{INK4A}$ protein expression (29).

$p16^{INK4A}$, the product of the *CDKN2* gene, one of the key cell cycle regulators, inhibits phosphorylation of the retinoblastoma protein, and thus acts as a negative regulator of the cell cycle (46). In contrast, loss of $p16^{INK4A}$ was found in only 1 of 5 tested apocrine carcinomas of the breast with loss $p16^{INK4A}$ protein expression, whereas the remaining 4 exhibited predominantly cytoplasmic patterns of distribution of the $p16^{INK4A}$ protein. Notably, several investigators previously reported a cytoplasmic distribution of the $p16^{INK4A}$ protein in a subset of breast carcinomas (47,48), which correlated with a high histological grade, loss of ER and PR, p53 protein over-expression and accelerated tumor proliferation, the parameters frequently featured in apocrine carcinoma and the MDA-MB-453 cell line. Similarly, the cell line exhibited a high cyclin D1 expression, which was not observed in the breast tissue samples.

In conclusion, the observed cytogenetic and molecular alterations in the MDA-MB-453 cell line were not consistently present in the apocrine tumor samples. Our cell culture results also differ in certain respects from previously published data. MDA-MB-453 is not an ideal model of apocrine carcinomas as recently defined using molecular methods. Notably, EGFR-positive apocrine carcinomas of the breast do not tend to harbor *K-RAS* gene mutations observed in the cell line, which indicates that targeted therapy with EGFR inhibitors may be a viable alternative in some patients.

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