

Sensitivity of Human Malignant Melanoma Cell Lines to Newcastle Disease Virus

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Abstract. *Background/Aim:* Virotherapy may be a promising alternative to chemotherapy of malignant melanoma. In clinical trials using strains of Newcastle disease virus (NDV), only a fraction of patients with cancer responded to virotherapy. In the present study, we tried to find a correlation between the susceptibility of human melanoma cell lines to NDV and growth factor signaling pathways. *Materials and Methods:* Using an ATP assay, cytotoxicity of an NDV strain (MTH-68/H) was tested in 13 human melanoma cell lines. The activation state of growth factor signaling pathways was studied by the analysis of key signaling proteins. *Results:* MTH-68/H was found to be cytotoxic in all melanoma cells tested, but the IC₅₀ values varied significantly. No correlation between the IC₅₀ values and the rate of extracellular signal-regulated kinase (ERK) and AKT phosphorylation and phosphatase and tensin homologue (PTEN) expression was found. *Conclusion:* Susceptibility of tumor cells to NDV may be affected by alterations other than those of RAS/ERK and phosphatidylinositol 3-kinase (PI3K)/AKT signaling in uninfected cells.

Malignant melanoma is an aggressive malignancy of melanocytes: it can be cured by surgical excision of the tumor in its early phase; metastasizing melanoma, however, is highly resistant to most conventional therapies and qualifies as one of the most deadly malignant diseases (1).

Recent advances in molecular pathology has identified several signaling pathways and regulatory circuits activated in melanoma cells (1-6). The most frequently activated

pathways in advanced melanoma are the RAS/extracellular signal-regulated kinase (ERK) pathway and the phosphatidylinositol-3-kinase (PI3K)/AKT pathway, up-regulated in 90% (2) and in 70% (5) of melanomas, respectively. Besides these, the retinoblastoma/p53, WNT, nuclear factor- κ B and microphthalmia-associated transcription factor signaling mechanisms (2), cell-cycle regulation, hypoxia signaling and cell adhesion are also often affected (3). The complexity of regulatory defects in melanomagenesis provides an explanation for the relative inefficiency of radio- and chemotherapeutic treatment protocols, and even attempts at targeted molecular therapies (6). Although drugs targeting key proteins of these signal transduction pathways hold promise of becoming effective anti-melanoma therapeutic agents, their pre-clinical and clinical testing is still in early phase (1, 3, 5, 6).

Oncolytic virotherapy has become an alternative approach to cancer treatment (7-9). Selective anti-tumor cytotoxicity of dozens of viruses, both natural and genetically engineered, have been tested in cell culture, and in animal and human studies (9). Out of the natural viruses, vesicular stomatitis virus (10), HF10 [an attenuated herpes simplex virus (11)], Coxsackievirus (12), Myxoma virus (13) and Newcastle disease virus (NDV) were found to be cytotoxic to melanoma cells. Viruses expressing a transgene *e.g.* adenovirus/interleukin 24 (*IL24*) (14), adenovirus/arrestin (15), vaccinia/ granulocyte macrophage-colony stimulating factor (*GM-CSF*) (16) NDV/*IL2*, NDV/*GM-CSF* and NDV/ tumor necrosis factor (*TNF*) (17) were also tested for melanoma cytotoxicity, with some success.

Unmanipulated, natural NDV was among the first viruses whose oncolytic potential was suspected (18). NDV is an avian paramyxovirus; its various strains are strongly pathogenic (velogenic), moderately virulent (mesogenic) or non-pathogenic (lentogenic) in birds, and are invariably non-pathogenic in humans (17). Its oncolytic effect may stem from two mechanisms: direct cytotoxicity causing apoptotic death of tumor cells, including melanoma cell lines (19); immunostimulatory effects leading to increased expression

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Key Words: Melanoma, oncolytic viruses, Newcastle disease virus, virotherapy.

of proteins involved in anti-tumor immune responses *e.g.* major histocompatibility complex, IFNs, chemokines (18, 20-22). Clinical trials using various NDV strains to treat patients with different types of advanced tumors indicated that only some patients had complete or partial response to NDV, while others were resistant to virotherapy (23-28). The reasons for the differences in susceptibility to NDV are currently unknown.

The aim of the present study was to determine the sensitivity of several human melanoma cell lines to MTH-68/H, an attenuated mesogenic NDV originating from the Herts'33 Hertfordshire strain (29). MTH-68/H is cytotoxic to rodent and human tumor cell lines of various tissue origin, it replicates in tumor but not in non-transformed cell lines, activates several signaling mechanisms in infected tumor cells [including IFN signaling, endoplasmic reticulum stress and apoptosis pathways (19)] accompanied by the up- or down-regulation of hundreds of genes (30). Among other tumor cell types, three human melanoma cell lines, HT-168-M1/9, HT199 and WM983B, were found to highly sensitive to MTH-68/H infection (19).

In the present study, 13 additional human melanoma cell lines were tested for MTH-68/H susceptibility by determining the 50% inhibitory concentration (IC_{50}) and 80% inhibitory concentration (IC_{80}) values using an ATP assay. Correlations between NDV sensitivity and the genetic status of key regulatory mechanisms (RAS/ERK pathway, PI3K/AKT pathway, and p53 protein) were sought.

Materials and Methods

Cell culture. Human melanoma cell lines UCSD-242L, SK-MEL-2, SK-MEL-5, SK-MEL-28, MALME-3M, UACC-62, UACC-257, and LOX-IMVI were obtained from the National Cancer Institute, Frederick National Laboratory for Cancer Research (Frederick, MD, USA), A375 (American Type Culture Collection (ATCC) cat. no. CRL-1619), A7 (CRL-2500), HMCB (CRL-9607), RPMI-7951 (HTB-66), and SK-MEL-3 (HTB-69) were purchased from the ATCC (Wesel, Germany). The Rat1 cell line (a gift from G. M. Cooper, Boston University) was used as control in the western blot experiments. UCSD-242L, SK-MEL-2, SK-MEL-5, SK-MEL-28, MALME-3M, UACC-62, UACC-257, LOX-IMVI cells were cultured in RPMI-1640 AQ media (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA). A375 cells were grown in Dulbecco's modified Eagle's medium (Sigma) containing 10% FBS. A7 cells were cultured in Eagles minimum Essential medium (EMEM) (Sigma) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 0.5 g/l geneticin (G418) and 10% FBS. HMCB cells were grown in EMEM containing 2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate and 10% FBS. RPMI-7951 cells were cultured in EMEM containing 2 mM glutamine, 1 mM sodium pyruvate and 10% FBS. SK-MEL-3 cells were grown in McCoy's 5A medium (Sigma) supplemented with 15% FBS.

ATP assay. Cells were plated onto white flat-bottom 96-well plates (Greiner, Frickenhausen, Germany) in triplicates. The optimal seeding

number for each cell line was determined individually in preliminary experiments: 2000 SK-MEL-2 cells; 1000 SK-MEL-5, MALME-3M, UACC-257, LOX-IMVI, and SK-MEL-3 cells; 750 HMCB cells; 500 UCSD 242L, SK-MEL-28, UACC-62, A7 and RPMI-7951 cells; and 250 A375 cells were plated. On the next day, cells were infected with different titers [multiplicities of infection (MOIs, particle/cell) 0.0005, 0.0025, 0.013, 0.064, 0.32, 1.6, 8, 40, 200, 1,000] of MTH-68/H for 72 h. For the positive control, cells were treated with 1 μ g/ml anisomycin, whereas for the negative controls, cells were treated with medium only. A luciferase-based ATP assay (Promega, Madison, WI, USA) was performed according to the manufacturer's instructions and luminescence was analyzed by FLUOstar Optima (BMG Labtech, Offenburg, Germany). IC_{50} and IC_{80} values were determined by Origin 8.0 (OriginLab, Guangzhou, China).

Western blotting. The preparation of protein extracts and the procedure of western blotting was performed as described elsewhere (31). Antibodies to phospho-ERK, phospho-AKT (Ser473), phosphatase and tensin homologue (PTEN) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling Technology (Beverly, MA, USA). Horseradish peroxidase conjugated anti-rabbit antibody was obtained from Pierce Biotechnology (Rockford, IL, USA).

Results and Discussion

The susceptibility of 13 human melanoma cell lines to the oncolytic NDV strain MTH-68/H was analyzed in this study. The IC_{50} and IC_{80} values of all cell lines were determined (Table I), and arbitrary categories of MTH-68/H sensitivity were established: <0.1 MOI, highly sensitive; 0.1-10 MOI, moderately sensitive; 10-1,000 MOI, weakly sensitive; >1,000 MOI, MTH-68/H-resistant. Using these criteria, five and eight melanoma cell lines were found to be highly and moderately MTH-68/H sensitive, respectively. The IC_{50} values covered a 200-fold range (from 0.013 to 2.57 MOI), giving an average of 0.548 MOI. The Rat-1 cell line used as control in this study was found to be resistant to MTH-68/H (19).

The aim of the present study was to determine the correlation between previously identified features of key melanomagenic genes (summarized in Table I), the state of activation of these signaling pathways in the melanoma cell lines (shown in Figures 1 and 2), and their susceptibility to NDV oncolysis.

Several signaling pathways and regulatory mechanisms are affected by mutations and gene expression alterations in melanoma. The most frequently affected pathway in the melanoma cell lines tested is the RAS/ERK pathway (12 out of 13 cell lines, no information was available for the A7 cell line; Table I): stimulation of this pathway may be caused by the activation/overexpression of growth factor receptors [point-mutation of epidermal growth factor receptor (*EGFR*) in SK-MEL-28, overexpression of the ephrin receptor *EPHA* in A375 and HMCB], activating NRasQ61R mutation (in SK-MEL-2), and *BRAF* mutation (in 10 cell lines). These 12 cell lines with activating mutations in signaling elements of the

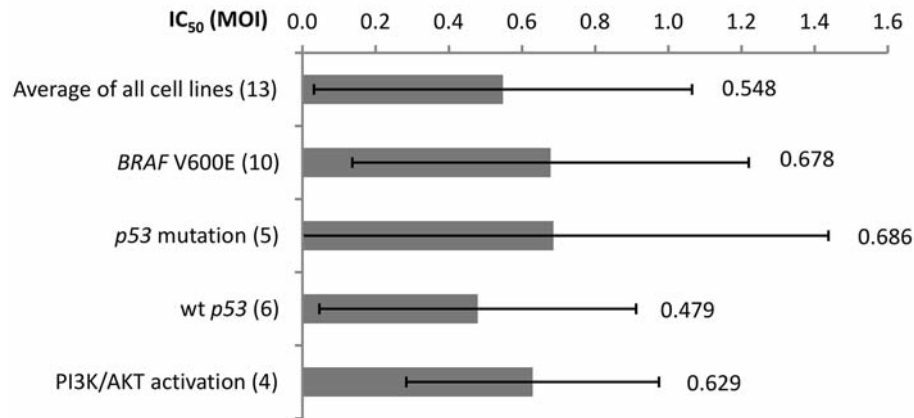


Figure 1. MTH-68/H sensitivity of various groups of human melanoma cell lines. The mean 50% inhibitory concentration (IC_{50}) values \pm SD were determined for the indicated groups of cell lines. Numbers in parentheses indicate the number of cell lines in the specific group.

RAS/ERK pathway, including the 10 cell lines carrying the BRAFV600E mutation, the most common genetic feature in melanoma, cover a wide range of MTH-68/H sensitivities (IC_{50} between 0.013 and 2.57 MOI).

In five of the cell lines, a mutant *p53* gene was identified (SK-MEL-2, SK-MEL-28, SK-MEL-3, HMCB, and RPMI-7951). The absence of *p53* mutation was demonstrated in another six melanoma cell lines (SK-MEL-5, MALME-3M, UACC-257, LOX-IMVI, A375 and UACC-62). There was no significant difference between the mean IC_{50} (0.686 and 0.479 MOI) and the range of susceptibility (0.013 to 2.57 MOI and 0.017 to 1.00 MOI, respectively). This is in agreement with our earlier findings that the function of *p53* protein is not required for the apoptotic effect of MTH-68/H on tumor cells (19).

Based on literature data (Table I) the PI3K/AKT pathway is expected to be constitutively active in SK-MEL-28 (*EGFR* mutation, *PTEN* mutation), HMCB, A375 (EPHA overexpression) and UACC62 (lack of *PTEN* expression). The average MTH-68/H sensitivity of these cells was not significantly different from the average for the 13 melanoma cell lines (Figure 1).

In order to determine the actual activation states of the RAS/ERK and PI3K/AKT pathways, western blot analysis of all the 13 melanoma cell lines using anti-phospho-ERK, anti-phospho-AKT and anti-PTEN was performed (Figure 2). Since the cell lines were arranged on the western blot according to their decreasing sensibility toward MTH-68/H, it is clearly apparent from Figure 2 that no correlation between ERK or AKT phosphorylation and NDV susceptibility exists. In addition, unexpectedly, the level of ERK or AKT phosphorylation did not always correlate with the genotype of the cell lines (see Table I). For example, although both MALME-3M and SK-MEL-3 cells carry the BRAFV600E mutant allele that codes for a

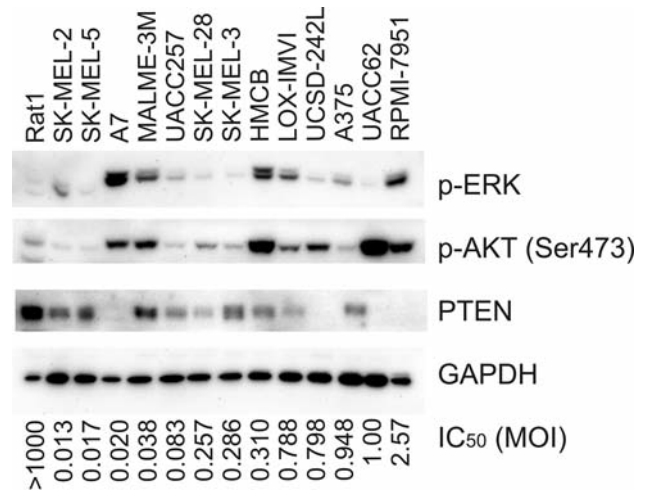


Figure 2. Extracellular signal-regulated kinase (ERK) and AKT phosphorylation and phosphatase and tensin homologue (PTEN) expression in individual cell lines. Western blot analysis of cell extracts using antibodies against phospho-(p)-ERK, p-AKT and PTEN was performed. Rat1 cells were used as controls. The melanoma cell lines were arranged in decreasing sensitivity to MTH-68/H according to the 50% inhibitory concentration (IC_{50}) value. Antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

constitutively active RAF kinase acting upstream of ERK kinases, ERK proteins were found to be highly phosphorylated and under-phosphorylated, respectively, in these cell lines. Similarly, SK-MEL-28 cell line carrying a mutated *PTEN* gene had a very low level of AKT phosphorylation (Table I, Figure 2). The *PTEN* tumor-suppressor protein acts upstream of AKT and thus an inverse relationship between *PTEN* expression and AKT phosphorylation is expected, and is in fact seen in most of

Table I. Genotypic and phenotypic features and susceptibility of human melanoma cell lines to Newcastle disease virus (NDV). Cell lines were cultured, infected with MTH-68/H at different multiplicities of infection (MOIs, particle/cell) and the 50% inhibitory concentration (IC₅₀) and 80% inhibitory concentration (IC₈₀) values were determined using an ATP assay as described in the Materials and Methods. Measurements were performed for each cell line in at least two independent experiments, in triplicates.

Cell line	Origin of cell line	IC ₅₀ (MOI)	IC ₈₀ (MOI)	Genotypic/phenotypic feature
<i>Highly sensitive to MTH-68/H</i>				
SK-MEL-2	Skin metastasis	0.013	0.058	<i>NRAS</i> Q61R [35] wt <i>BRAF</i> [35,36] No constitutively active AKT [33] p53 G245S [37]
SK-MEL-5	Axillary node metastasis	0.017	0.065	wt <i>NRAS</i> [35] <i>BRAF</i> V600E [35, 36] wt <i>p53</i> [38]
A7 (M2A7)	ABP (filamin-1)-transfected M2 skin melanoma cell line	0.020	0.363	
MALME-3M	Lung metastasis	0.038	0.155	wt <i>NRAS</i> [35, 40, 42] <i>BRAF</i> V600E [35, 38-40] wt <i>p53</i> [37-40] wt <i>PTEN</i> [40, 42] Weakly invasive [41]
UACC-257	Malignant melanoma	0.083	0.370	wt <i>NRAS</i> [35, 40] <i>BRAF</i> V600E [35,40] High phospho-ERK [32] wt <i>p53</i> [38, 40] wt <i>PTEN</i> [40]
<i>Moderately sensitive to MTH-68/H</i>				
SK-MEL-28	Malignant melanoma	0.257	0.922	<i>EGFR</i> P753S [40] wt <i>NRAS</i> [35, 40, 42] <i>BRAF</i> V600E [35, 36, 39, 40] High phospho-ERK [32] <i>p53</i> L145R [37, 39, 40] <i>PTEN</i> T167A [42], Low <i>PTEN</i> expression [43] Constitutively active AKT [33] Weakly invasive [41]
SK-MEL-3	Lymph node metastasis	0.286	1.80	wt <i>NRAS</i> [40] <i>BRAF</i> V600E [36, 40] <i>p53</i> R267W [40] wt <i>PTEN</i> [40]
HMCB	Bowes melanoma	0.310	6.10	High EPHA expression [44] wt <i>BRAF</i> [39] <i>p53</i> H193R [39]
LOX-IMVI	Lymph node metastasis, amelanotic	0.788	6.00	wt <i>NRAS</i> [35, 40] <i>BRAF</i> V600E [35, 40] wt <i>p53</i> [38, 40] wt <i>PTEN</i> [40]
UCSD-242L	Malignant melanoma	0.798	2.25	Strongly invasive [41] wt <i>NRAS</i> [35] <i>BRAF</i> V600E [35]
A375	Primary melanoma	0.948	4.43	High EPHA expression [44] wt <i>NRAS</i> [35, 40, 42] <i>BRAF</i> V600E [35, 36, 39, 40] High phospho-ERK [32] wt <i>p53</i> [37, 38, 40] wt <i>PTEN</i> [40, 42]
UACC-62	Malignant melanoma	1.000	2.46	Strongly invasive [41] wt <i>NRAS</i> [35, 40] <i>BRAF</i> V600E [35, 40] wt <i>p53</i> [38,40] <i>PTEN</i> P248fs [40], No <i>PTEN</i> expression [43]
RPMI-7951	Lymph node metastasis	2.565	8.80	wt <i>NRAS</i> [35] <i>BRAF</i> V600E [35, 39] <i>p53</i> S166X [37, 39] Strongly invasive [41]

the melanoma cell lines (Figure 2). These observations suggest that the activation state of these mitogenic/pro-survival pathways is affected by features other than those that have already been identified (*e.g.* the level of expression of the activated BRAF protein, activity and level of specific phosphoprotein phosphatases, cross-talk between signaling pathways *etc.*). In addition, some of our western blot results contradict observations published earlier. For example, SK-MEL-28 cells were reported to contain highly phosphorylated ERK (32) and AKT (32, 33) protein kinases. In contrast, we found both enzymes to have phosphorylation states comparable in SK-MEL-28 and non-transformed Rat1 cells (Figure 2). The reason for this discrepancy is not clear; it may reflect individual differences between different batches of the same cell line or differences in cell-culture conditions.

The results presented herein indicate that high basal activity of the ERK and AKT pathway is not required for the oncolytic action of MTH-68/H, SK-MEL-5, for example, while being highly susceptible, displayed very low levels of both ERK and AKT phosphorylation. On the other hand, overactive ERK and AKT pathways (as in RPMI-7951 cells) are not sufficient to provide high NDV susceptibility. In contrast to our findings with NDV, oncolysis by reovirus requires an activated RAS pathway that is essential to stimulate the synthesis of viral proteins (34). In NDV-infected melanoma cells, activation of this pathway alone does not determine the relative susceptibility of melanoma cell lines toward the virus. The lack of correlation between MTH-68/H sensitivity and the state of ERK and AKT signaling may be interpreted in several ways. Firstly, hundreds of cellular proteins may be involved in the process of viral oncolysis and their basal levels or activation states may be more important in NDV sensitivity than activation of ERK and AKT signaling. Secondly, it may be the response of these proteins to NDV infection rather than their basal state that matters. It may be of importance that MTH-68/H infection of PC12 rat pheochromocytoma cells leads to the induction and repression of hundreds of genes (30). The products of the affected genes are involved in diverse cellular processes (*e.g.* IFN signaling, apoptosis, anti-viral responses, endoplasmic reticulum stress, and cell-cycle regulation) that may have a role during NDV infection. Differences in NDV induction/repression patterns of these genes may correlate with the sensitivity of cell lines to NDV infection. Systematic analysis of key signaling proteins of these regulatory mechanisms may lead to identification of useful markers of NDV susceptibility.

Acknowledgements

This work was supported by Eden Therapeutics, Houston, Texas, USA and by Science, Please! Research Team on Innovation (grant number SROP-4.2.2/08/1/2008-0011).

The present scientific contribution is dedicated to the 650th anniversary of the foundation of the University of Pécs, Hungary.

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Received May 26, 2015

Revised July 8, 2015

Accepted July 13, 2015