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Her2/neu as a novel therapeutic target in sinonasal undifferentiated carcinoma

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Abstract

Background: Sinonasal undifferentiated carcinoma (SNUC) is a rare and aggressive cancer. Despite multimodal therapy, the prognosis in SNUC remains poor, and new therapies are needed. Thus, we explored potential therapeutic targets in SNUC.

Methods: Using the human-derived SNUC MDA8788–6 cell line, we performed whole genome single nucleotide polymorphism analysis to identify copy number changes in this line. Protein expression levels were evaluated by Western blotting. Cell growth inhibition was assessed by MTT and clonogenic assays. The mouse flank model was used to examine the effect of growth inhibition *in vivo*.

Results: The *ERBB2* gene was highly amplified and cell extracts showed HER2 was overexpressed and phosphorylated in MDA8788–6. Lapatinib effectively inhibited HER2 signaling pathway in our SNUC cell line. HER2 inhibition successfully suppressed the cell growth of MDA8788–6 cells both *in vitro* and *in vivo*.

Conclusions: Targeting HER2 may be promising avenue for the development of novel therapies for SNUC.

Keywords

Sinonasal Undifferentiated Carcinoma; gene amplification; HER2/neu; molecular targeted therapy; apoptosis

Introduction

Sinonasal undifferentiated carcinoma (SNUC) is a rare, highly aggressive cancer that arises in the nasal cavity and paranasal sinuses. Initially described by Frierson, et al. in 1986⁽¹⁾,

this tumor is a neuroendocrine sinonasal malignancy, a category that includes esthesioneuroblastoma, neuroendocrine carcinoma, and small cell carcinoma. In general, SNUCs present as large tumors that involve multiple sinonasal structures and often extend into the orbit or cranial cavity; these tumors can metastasize to the cervical lymph nodes, lungs, bones, brain, and liver (2–5). The treatment of SNUC includes aggressive multimodal therapy with radiotherapy and chemotherapy and in some instances, surgery (3, 5–7). Despite aggressive management of SNUC, the prognosis remains poor, with a median survival time from diagnosis of ranging 15 to 58 months (6, 8–11). Thus, development of new therapies is essential to improve survival in patients with SNUC.

Molecular targeted therapies represent a promising option for development of novel therapies in SNUC. While traditional or conventional chemotherapies simply interfere with all rapidly dividing cells, molecular targeted therapies block the growth of cancer cells by interfering with specific, targeted molecules needed for tumor growth. One well-known example of a targeted therapy for breast cancer is targeting human the Epidermal Growth Factor Receptor 2 (HER2), also known as Neu, encoded by the *ERBB2* gene, which is overexpressed in approximately 20–30% of breast cancers (12–15).

In our previous study, we reported the establishment and characterization of the first human SNUC cell line (16). In the current study, we determined whether HER2 is a potential therapeutic target in SNUC using in vivo and in vitro approaches.

Materials and Methods

Surgical Specimen, Cell lines and cell culture

Human SNUC cell line MDA8788–6 was established and cultured previously (16). Briefly, a resected specimen obtained from a female patient with T4N0M0 SNUC was maintained and expanded in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies Corporation, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO) and 0.1 mg/mL of Primocin (InvivoGen, San Diego, CA). Human squamous cell carcinoma (SCC) cell line derived from the maxillary sinus UMSCC33 was obtained from Dr. Thomas Carey (University of Michigan) in July 2009, and another SCC line derived from the oral cavity was obtained from Dr. Zhen Fan at our institute in September 2008. Breast cancer cell line SKBR3 was provided by Dr. Dihua Yu at MD Anderson in April 2009, and human umbilical vein endothelial cell (HUVEC) line was purchased from ScienCell Research Laboratories (San Diego, CA) in September 2012. UMSCC33, HN5, and SKBR3 were grown on tissue culture dishes in DMEM containing 10% FBS. HUVEC was cultured in Medium 200PRF with Low Serum Growth Supplement (Life Technologies Corporation). The identity of all cell lines was authenticated using short tandem repeat testing within 6 months of cell use.

Reagents

Lapatinib ditosylate was purchased from LC Laboratories (Woburn, MA). Trastuzumab (Genentech, Inc., South San Francisco, CA) was obtained from our pharmacy at MD Anderson.

Whole Genome SNP Analysis

Whole genome SNP analysis of MDA8788–6 cells was performed using the methodologies described previously⁽¹⁷⁾. Briefly, whole genome DNA from MDA8788–6 cells and cancer associated fibroblast derived from the same donor were isolated by using ArchivePure DNA Purification kit (5 PRIME, Gaithersburg, MD). The sample was analyzed on the Affymetrix Human SNP6.0 array (Affymetrix, Santa Clara, CA) at the MD Anderson DNA Core Facility and the data was processed and analyzed using commercial software Partek® Genomics Suite™ (<http://www.partek.com>) and R packages (Partek Inc., Saint Louis, MO). The image was taken as a screenshot in Integrative Genomics Viewer (IGV, Broad Institute, Cambridge, MA).

Protein Analysis

The cells from all lines (MDA8788–6, UMSCC33, HN5, HUVEC, and SKBR3) were lysed in 1x Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA) with protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, PhosSTOP, Complete protease inhibitor cocktail) (Roche Diagnostics Corporation, Indianapolis, IN). Organ homogenates in 2x Cell Lysis Buffer with appropriate inhibitors were clarified by centrifugation (total lysate). The following antibodies were obtained from respective suppliers as follows: phospho-erbB-2/HER-2 (Tyr1248) (EMD Millipore, Billerica, MA), c-erbB-2 (clone 3B5), c-Kit (clone28) (BD Biosciences, San Jose, CA), PARP, EGFR, VEGFR2 (55B11), phospho-AKT (Thr 308), pan-AKT (40D4), phospho-ERK (p44/42 MAP Kinase) (Thr202/Tyr204), ERK (p44/42 MAP Kinase), PDGFRβ (Cell Signaling Technology), and β-actin clone AC-15 (Sigma-Aldrich).

Clonogenic Survival Assay

To determine the sensitivity of the MDA8788–6 cell line to lapatinib, we performed a clonogenic survival assay using the SKBR3 cell line as the control. One thousand MDA8788–6 cells or SKBR3 cells were plated on 6-well plates. Twenty-four hours later, cells were treated with serially diluted lapatinib (dimethyl sulfoxide [DMSO] control, 0.25, 0.5, 1.0, 2.0, and 4 μM) for 48 hours. After 10 days of incubation, the cells were stained with 0.5% crystal violet in 10% formaldehyde and their images were captured by an HP Scanjet G4010 Photo Scanner (Hewlett-Packard Company, Palo Alto, CA). Colonies were counted with Image J (National Institute of Health, Bethesda, MD), and the surviving fraction was calculated using the following equation: (number of colonies counted)/ (number of cells seeded x plating efficiency/100).

Cell Proliferation Assay

The anti-proliferative effect of lapatinib on the SNUC cells *in vitro* was determined using an methyl thiazolyl tetrazolium (MTT) assay as we previously described⁽¹⁸⁾. Briefly, MDA8788–6 and SKBR3 cells were plated in 96-well plates in medium. After a 24-hour attachment period, cells were incubated for 72 hours in various concentration of lapatinib (0.5 nM–30 μM) or DMSO alone as a control. Then cells were incubated for 3 hours in medium containing 0.25 mg/mL 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich). The cells were lysed in 100 μL DMSO to release formazan. We

used an EL-808 96-well plate reader (BioTek Instruments, Inc., Winooski, VT) and set at an absorbance of 570 nm to quantify the conversion of MTT to formazan. The concentration of lapatinib achieving 50% growth inhibition (GI50) for each cell line was calculated using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA). The experiment was repeated three times.

TUNEL Assay

To detect apoptotic cells, terminal deoxynucleotidyl-mediated dUTP Nick End Labeling (TUNEL) assay was performed using In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics Corporation) according to the manufacturer's instructions. Briefly, 2×10^4 cells were seeded on a poly-L-lysine coated coverslip (Corning, Inc., Corning, NY). Twenty four hours after seeding, the cells were treated with 2 μ M of lapatinib for 48 hours. After washing with phosphate buffered saline (PBS) three times, the cells were fixed in 2% paraformaldehyde solution in PBS. The cells were labeled following manufacturer's instructions and were observed under a fluorescence microscope. The MDA8788-6 cell line treated with 1 μ M staurosporine (Thermo Fisher Scientific, Waltham, MA) for 7 hours served as a positive control.

Animal Care

Male nude mice (aged 6–8 weeks) were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). The mice were maintained in a pathogen-free environment and fed irradiated mouse chow and autoclaved, reverse osmosis-treated water at facilities in accordance with current regulations and standards of the U.S. Department of Agriculture, U.S. Department of Health and Human Services, and the National Institutes of Health. The mice were used in accordance with MD Anderson's Animal Care and Use Guidelines, and all animal procedures were done in accordance with a protocol approved by the institution's Institutional Animal Care and Use Committee.

Subcutaneous Flank Model

A subcutaneous flank nude mouse model was established by injecting 2×10^6 MDA8788-6 cells suspended in a volume of 200 μ L of PBS into the flank of mice as described previously⁽¹⁶⁾. Six days after the injection, the mice were randomly assigned to two groups; control (PBS) and treatment (trastuzumab) groups. Trastuzumab was administrated intraperitoneally at a concentration of 8 mg/kg in sterile PBS twice per week for 5 weeks. Control mice were given sterile PBS intraperitoneally twice per week for 5 weeks. Mice were examined twice weekly for tumor development. Tumors were measured using calipers in cephalad-to-caudad and left-to-right dimensions. Tumor volume was calculated as $V = AB^2 (\pi/6)$, where A is the longest dimension of the tumor and B is the dimension of the tumor perpendicular to A . The mice were euthanized with carbon dioxide at day 48 after the initial inoculation. At the time of death, the tumors and lungs were harvested and placed in 10% buffered formalin solution overnight for fixation, and were embedded in paraffin for histological analysis.

Immunohistochemistry

Sections from paraffin-embedded tumors were stained with hematoxylin-eosin (H&E). In addition, immunohistochemical analysis of tissue sections for Ki-67 (Clone MIB-1, Dako North America, Inc., Carpinteria, CA) was conducted as previously described ⁽¹⁹⁾.

Statistical Analysis

The unpaired 2-tailed t test was used to compare the differences in mean tumor volume and Ki-67 positive cells between 2 groups (PBS and trastuzumab) with GraphPad Prism 5. *P* values < 0.05 were considered statistically significant.

Results

HER2 expression in MDA8788–6 cells

To explore whether the genes encoding any targetable tyrosine kinase receptors for the treatment of SNUC are amplified, we performed whole SNP analysis on MDA8788–6. Cancer associated fibroblasts derived from the patient from whom we established the human SNUC cell line MDA8788–6 served as a reference. The analysis demonstrated that the q12 region of chromosome 17, where the *ERBB2* gene is located, was highly amplified (Fig. 1A). Then we examined whether the *ERBB2* gene amplification resulted in overexpression of HER2 protein in the MDA8788–6 cell line, using the SKBR3 cell line as a HER2 amplification/overexpression control ⁽²⁰⁾, Western blot analysis of both MDA8788–6 cells and the original tissue from the patient showed high level of phosphorylation of HER2 as well as total HER2 expression (Fig. 1B). We also screened the MDA SNUC cell line for expression of other tyrosine kinase receptors (EGFR, platelet-derived growth factor receptor beta polypeptide [PDGFRβ], c-Kit and vascular endothelial growth factor receptor 2 [VEGFR2]). Both the MDA cell line and the surgical specimen demonstrated minimal expression any of these receptors (Fig. 1C).

Down-regulation of HER2 signaling pathway by lapatinib in MDA8788–6 cells

Since the MDA8788–6 cell line overexpressed phospho-HER2 and HER2, we sought to investigate the effect of HER2 receptor inhibition with the dual small molecule EGFR/HER2 inhibitor, lapatinib ⁽²¹⁾. This inhibitor has been applied in the treatment of breast cancer and other HER2 positive cancers. Exposure of MDA8788–6 and SKBR3 to 2 μM lapatinib, a clinically achievable dose ⁽²¹⁾, led to significantly decreased levels of phospho-HER2 one hour after administration and this decrease was sustained up to 24 hours after administration (Fig. 2A). HER2 phosphorylation was also down-regulated in a dose dependent manner; HER2 was significantly dephosphorylated by lapatinib at as low a dosage as 0.2 μM and was almost completely unphosphorylated at 1–2 μM in both cell lines (Fig. 2B). The PI3K/AKT and MAPK/ERK pathways, which are the major downstream pathways of HER2 ⁽²²⁾, were also phosphorylated at significantly lower levels of after lapatinib treatment in dose dependent and time dependent manners (Fig. 2A and 2B). These results indicated that lapatinib treatment successfully down regulated the phosphorylation of HER2 and its downstream signaling kinases AKT and ERK.

Survival and growth suppression by lapatinib in MDA8788–6 cells

Because lapatinib efficiently blocked the HER2 signaling pathways, we evaluated whether lapatinib could suppress growth and survival under *in vitro* conditions. The clonogenic survival assay revealed that lapatinib's potent inhibition of survival in both the control SKBR3 and the MDA8788–6 cell line at clinically relevant doses (IC₅₀: 0.3487 μ M and 0.1168 μ M respectively) (Fig. 3A). These data demonstrated that the blockage of the HER2 signaling pathways by lapatinib contributed to decreased clonogenic survival and growth suppression.

Induction of apoptosis by lapatinib in MDA8788–6 cells

In order to determine the mechanisms of cell survival/growth inhibition by lapatinib, we explored whether treatment with this agent leads to apoptosis of SNUC cells. To identify apoptotic cell death, the TUNEL assay was performed to detect DNA fragmentation. Treating the SNUC cells with 2 μ M lapatinib for 48 hours showed positive TUNEL staining suggesting that some cells underwent apoptosis (Fig. 4A). The cleaved PARP fragment, another marker for apoptosis, was induced after 24–72 hours of lapatinib treatment, as revealed by Western blotting analysis (Fig. 4B). Both results indicated that blocking the HER2 signal pathways by lapatinib induced apoptotic cell death in the SNUC cell line.

Mechanisms of apoptosis by lapatinib in the MDA8788–6 cells

To determine which molecules are involved in the cell death caused by lapatinib in the SNUC cell line, additional pro-apoptotic and pro-survival markers were analyzed by Western blotting. While the expression of pro-apoptotic protein Bim was induced by lapatinib, cleaved caspase-3 was not induced by lapatinib even after 72 hours (Fig. 5A). On the other hand, lapatinib effectively suppressed expression of pro-survival XIAP, although another pro-survival protein Mcl-1 was not affected by lapatinib (Fig. 5B). These results suggested that the SNUC cells underwent apoptosis through induction of pro-apoptotic molecules and suppression of pro-survival molecules.

Tumor growth inhibition *in vivo* by blocking HER2

Since the inhibition of HER2 signal pathway successfully suppressed cell growth of the MDA8788–6 SNUC cells, we examined whether blocking HER2 could also affect tumor growth *in vivo*. Six days after the inoculation with SNUC cells, mice were treated with PBS or a humanoid monoclonal anti-HER2 antibody, trastuzumab, which has been used for the treatment of HER2-positive breast cancer. While tumors in the vehicle control treated mice grew steadily trastuzumab significantly reduced the size of tumors by day 31 (PBS) ($P < 0.0001$) (Fig. 6A, 6B, and Supplementary Fig. A and B). As expected, sections from the treatment group showed tumor destruction by H&E staining (Fig. 6C) and lower proliferation rate by Ki-67 staining (Fig. 6D and 6E) compared to ones from the control group. This animal study also supported efficacy of HER2 inhibition treatment for SNUC. This study indicated that HER2 inhibition also successfully suppressed tumor growth *in vivo*.

Discussion

The aim of this study was to explore novel targeted therapies for SNUC, which is a rare and highly aggressive cancer. In this study, we found that lapatinib effectively inhibited the HER2 signaling pathway in our SNUC cell line. HER2 inhibition successfully suppressed the cell growth of MDA8788–6 cells both *in vitro* and *in vivo*.

Conventional chemotherapeutic agents that have been employed for the treatment of SNUC include doxorubicin, cyclophosphamide, vinblastine, etoposide, and cisplatin ⁽⁵⁾. However, the prognosis for patients with SNUC is still poor, and therefore, development of molecular targeted therapies in SNUC is critically important. While various small-molecule inhibitors or humanized antibodies have been using to inhibit function of tyrosine kinases including tyrosine kinase receptors (TKRs) in some types of cancers, few targetable molecules have been identified in SNUC ⁽²³⁾. Another important aspect of using inhibitors is that some mutations in genes of interest affect the responses to tyrosine kinase inhibitors. For example, small molecule inhibitors of erlotinib and gefitinib, which are FDA-approved drugs for the treatment of non-small cell lung carcinoma, are more effective for patients whose tumors have activating mutations in the tyrosine domain of the *EGFR* gene ⁽²⁴⁾. In attempt to identify clinically relevant activating mutations in SNUC, we previously analyzed hot spot mutations within well-characterized 12 oncogenes or tumor suppressor genes (BRAF, KRAS, beta-catenin, EGFR, VEGFR, c-KIT, PDGFR A, FBXW7, PI3K, AKT, JAK2, and CDK4) in 13 SNUC cases ⁽²⁵⁾, but did not identify any activating mutations in this study.

Amplification or overexpression of the *ERBB2* gene occurs in about 20–30% of breast cancers ^(12–15) and 7–34 % of gastric cancers ^(26–28), and targeting HER2 has been applied in the treatment of patients with these tumor types. In this study, we demonstrated that the *ERBB2* gene was highly amplified and the HER2 protein was over expressed and phosphorylated in the MDA8788–6 SNUC cell line. Overexpression of HER2 was also confirmed in the original surgical specimen, suggesting that the amplification of HER2 did not occur during establishment of the cell line. On the other hand, the amplification of other growth factor receptors found in other types of tumors, such as EGFR ⁽²⁹⁾, PDGFR β , c-Kit and VEGFR2 were not observed in this cell line (data not shown) and their protein products were not found to be overexpressed.

Based on our studies we were interested in whether inhibition of the HER2 receptor could suppress growth of the SNUC cells both *in vitro* and *in vivo*. As we expected, HER2 inhibition successfully suppressed cell growth and also down-regulated PI3K/AKT and MEK/MAPK and activated pro-apoptotic pathways. Interestingly, lapatinib down-regulated the pro-survival protein XIAP in the MDA8788–6 SNUC cells but not in the HER2-amplified breast cancer SKBR3 cells, suggesting that the mechanisms of inducing apoptosis by lapatinib may differ from one cell line to another.

It is important to know which SNUC patients would benefit from HER2 targeted therapy. So far, the over-expression of HER2 in SNUC has yet to be reported. According to a small study analyzing 11 SNUC cases conducted by Chornock et al., none of the cases were positive for HER2 (One case was 1+, weak, and two cases were 2+, weak) by

immunohistochemistry (IHC) ⁽²³⁾. Our specimens used for establishing the SNUC cell line showed a negative result for HER2 staining by IHC (data not shown) whereas Western blotting showed strong expression of HER2 protein indicating that the conventional HER2 immunohistochemical staining may not be the best way to evaluate the status of HER2 in SNUC specimens. Another example of this is seen in gastric cancer, where amplification of the *ERBB2* gene is detected by in situ hybridization even in some HER2 negative or equivocal cases by IHC ^(26, 30). Introducing *HER2* FISH testing to SNUC cases might be a good idea to select patients for HER2 targeted therapy.

Taken together, our results suggest that targeting HER2 may be promising avenue for the development of novel therapies for SNUC. To the best of our knowledge, this is the first report of molecular-targeted therapy in SNUC. Our next step is to identify more targetable molecules by conducting whole genome analysis to develop more efficient strategies for SNUC treatment as well as to study the impact of HER2 targeting in combination with chemotherapy and or radiotherapy on SNUC growth and survival.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: Amplification of the *ERBB2* gene and expression of HER2 protein in MDA8766 cells. (A) Whole SNP analysis of MDA8788–6 cells showed amplification of 17q12 where the *ERBB2* gene is located. (B) Protein expression and phosphorylation levels of HER2 were analyzed by Western blotting using SKBR3 as a positive control. UMSCC33 is a squamous cell carcinoma line derived from the maxillary sinus and used as a negative control. Both MDA8788–6 and the surgical specimen that the cell line was isolated from showed strong expression of phosphorylated-HER2 (Tyr 1248) and total HER2 protein. (C) Expressions of other growth factor receptors (EGFR, PEGFRβ, VEGFR2, and c-Kit) in MDA8788–6 cells and the original tumor were examined by Western blotting. Neither the SNUC cell line nor the tissue from the patient with SNUC expressed any of these receptors. Cell lysate from HN5, CAF, HUVEC, and HEL served as positive controls of these growth factor receptors.

Figure 2: Down-regulation of HER2 signaling by lapatinib in MDA8788-6 cells.

HER2-overexpressing SKBR3 and MDA8788-6 cells were treated with the EGFR/HER2 inhibitor lapatinib. Exposure to lapatinib led to lower levels of phosphorylated HER2 and its downstream target molecules, AKT and ERK in a (A) time dependent manner (2 μ M, 1~24 hours) and (B) dose dependent manner (0.1~2 μ M, 24 hours) in both cell lines.

Figure 3: Suppression of cell survival and cell growth in MDA8788-6 cells by lapatinib.

(A) Clonogenic survival assay of MDA8788-6 cells using SKBR3 as a positive control. After 24 hours of plating, cells were treated with various concentrations of lapatinib for 48 hours. The cells were incubated another 10 days and stained with crystal violet. Potent inhibition of survival by lapatinib was observed in both the control SKBR3 and the SNUC cell line at clinically relevant doses. (B) Robust cell growth inhibition was also observed by MTT assay at low concentrations of lapatinib in both SKBR3 and MDA8788-6.

A **B**

Figure 4: Induction of apoptosis by lapatinib treatment in MDA8788-6 cells.

(A) Apoptotic cells were detected by TUNEL and labeled with green fluorescent color after 48 hours incubation with 2 μ M lapatinib. The MDA8788-6 cell line treated with 1 μ M staurosporine for 7 hours served as a positive control and treatment with the TUNEL reaction mixture omitting Enzyme solution served as a negative control. (B) PARP cleavage was shown by Western blotting after 24–72 hours of lapatinib treatment. Whole cell lysate from MDA8788-6 treated with staurosporin served as a positive control.

Figure 5: Detection of pro- and anti-apoptotic proteins' response to lapatinib in MDA8788-6 cells.

(A) Lapatinib's induction of pro-apoptotic protein Bim was observed in both SKBR3 and MDA8788-6 cells. On the other hand, cleaved caspase-3 was not induced by lapatinib even after 72 hours. (B) The pro-survival protein XIAP showed decrease expression after treatment of cells with lapatinib, whereas the levels of another pro-survival protein, Mcl-1, were not affected by lapatinib.

Figure 6: Suppression of the growth of tumor xenografts by trastuzumab treatment.

(A) 2×10^6 MDA8788-6 cells were injected into the flank of 20 mice. After 6 days of the inoculation, the mice were randomized to a treatment group and a control group; the treatment group was given 8 mg/kg trastuzumab twice per week intraperitoneally and the control group was given phosphate buffered saline (PBS) twice per week. While the control group tumors grew steadily after, trastuzumab treatment significantly reduced the size of tumors by day 31 compared to the vehicle control (PBS) ($P<0.0001$). (B) Appearance of treated subcutaneous MDA8788-6 tumors in nude mice. (C) H&E shows tumor shrinkage in the treatment group. (D and E) There were significantly fewer Ki67-positive cells in tumors from the trastuzumab treated group than from the control group ($P=0.0068$).