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ORIGINAL ARTICLE

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# Expression of HER2/neu is uncommon in human neuroblastic tumors and is unrelated to tumor progression

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**Abstract** Neuroblastic tumors (NT) are the most frequently occurring extracranial solid tumors during childhood. The overall 5-year survival is approximately 20% for patients with metastatic disease. Novel treatments are therefore intensively sought and tumor-targeted immuno- and chemotherapy appear promising. The HER2/neu oncogene, which is highly homologous to the EGF receptor, was initially isolated from rat neuroblastoma cells. HER2/neu over-expression is frequently detected in breast tumors and constitutes an important unfavorable prognostic factor. HER2/neu is a suitable target for antibody-based immunotherapy, as demonstrated by the clinical efficacy of the Herceptin monoclonal antibody (mAb), which reacts with its extracellular domain. Expression of HER2/neu has also been reported to be a negative prognostic factor in a small survey of NT tumors. Here, we have investigated HER2/neu expression in 14 human and 2 murine neuroblastoma (NB) cell lines by flow cytometric analysis and in 93 NT by means of a certified immunohistochemical system. HER2/neu over-expression was found in 2 human cell lines and 11 tumors (14% for both types of samples). No significant association was found between HER2/neu expression and stage, age, sex, ploidy, histological type or subtype. Moreover, log rank test indicated that overall and event-free survival was not significantly different in HER2/neu positive and negative patients. These data suggest that HER2/neu should not be considered as a relevant prognostic factor in NT, and that HER2/neu-based immunotherapy may be feasible only in a minority of NT patients.

**Keywords** Cancer · c-erb2 · HER2/neu · Immunotherapy · Neuroblastoma

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## Introduction

Neuroblastic tumors (NT) are the most frequently occurring extracranial solid tumors during childhood. The overall 5-year survival for patients with metastatic disease is approximately 20%, despite the use of myeloablative therapy followed by autologous hematopoietic stem cell rescue. Novel treatments are intensively sought, as well as effective methods to eliminate contaminant tumor cells from bone marrow or peripheral blood stem cell grafts.

Antibody-mediated immunotherapy, antibody-targeted chemotherapy and tumor-specific adoptive immunotherapy appear to be the most promising new therapeutic approaches. Phase I/II trials with anti-$GD_2$ antibodies have provided encouraging results [9, 14, 19]. However, the identification of additional NT-associated antigens might increase the spectrum of potential therapeutic targets.

The HER2/neu oncogene – human epidermal growth factor-related gene-2 receptor – was initially isolated from an ethylnitrosurea-induced rat neuroblastoma [26] and HER2/neu expression has been reported to be a negative prognostic factor in a small survey of NT [15]. HER2/neu represents an important negative prognostic factor in breast tumors [20], and its over-expression has been frequently found in a variety of human cancers, such as non-small cell lung carcinoma [13], pancreatic

adenocarcinoma [24], salivary gland carcinoma [23], thymoma [10], prostate [7], gastric [28], colonic [8], uterine [25], and ovarian [5] cancer. In many of these tumors HER2/neu over-expression correlated with advanced stage and worse outcome.

HER2/neu represents an actractive target for antibody-mediated immunotherapy and antibody-targeted chemotherapy. Trastuzumab (Herceptin; Genentech, San Francisco, Calif.), a humanized monoclonal antibody (mAb) which recognizes the HER2/neu extracellular domain, has proven effective in breast cancer patients with documented over-expression of HER2/neu in the malignant cells [16]. Trastuzumab is endowed with an intrinsic anticancer activity when administered as a single agent, and synergizes with cytotoxic drugs such as paclitaxel and doxorubicin [3]. Recently, a single-chain Fv fragment recognizing HER2/neu has also been produced and validated [29]. This recombinant molecule may allow tumor-specific targeting of therapeutic agents thanks to its improved biodistribution. In addition, HER2/neu peptides, derived from the HER2/neu protein and presented by dendritic cells to $CD4^+$ lymphocytes in the context of HLA-DR molecules, have been shown to give rise to specific T cell immunity and consequent production of specific antibodies [22]. Finally, bispecific anti HER2/neu antibodies [17] or immunotoxin conjugates [27] may be useful tools for eliminating contaminant tumor cells from grafts.

On the basis of the above-mentioned factors, we have evaluated HER2/neu over-expression in a large series of NB cell lines and NT in order to assess the feasibility of HER2/neu-based immunotherapy in NT patients. Only 14% of neuroblastic tumors and cell lines were found to over-express HER2/neu, clearly indicating that HER2/neu-based therapeutic approaches are not feasible in most NT patients.

## Materials and methods

### Flow cytometric analysis of NB cell lines

The NB cell lines used in this study were: ACN (kindly provided by S. Carrel, Lausanne, Switzerland), GI-CA-N, GI-LI-N, GI-ME-N (established at the Laboratory of Oncology, Gaslini Institute, Genoa, Italy), IMR-32, SK-N-SH and Neuro-2a (obtained from ATCC, Rockville, Md.), LAN-1, LAN-5 (kindly provided by R. Seeger, Los Angeles, Calif.), SK-N-BE(2) and SK-N-BE(2)c (kindly provided by J. Biedler, New York, N.Y.), NXS2 (kindly provided by Dr. Reisfield, Scripps Clinic, La Jolla, Calif.), IMR-5, SK-N-FI, SK-N-AS, SH-SY-5Y (obtained from ECACC, Genoa, Italy). Cells were cultured in RPMI 1640 or DMEM medium (BioWhittaker, Caravaggio, Italy) supplemented with 10% FCS (Biochrom-Seromed, Berlin, Germany), glutamine and penicillin-streptomycin (BioWhittaker) in a 5% $CO_2$ atmosphere at 37°C. Surface expression of HER2/neu was tested by indirect immunofluorescence and cytofluorimetric analysis using OP 14 mAb from Oncogene Research (Merck, Darmstadt, Germany) as first step and a FITC-conjugated goat anti-mouse Ig (Immunotech, Marseille, France) as second step reagent. Control was an isotype-matched murine mAb of irrelevant specificity. Samples were analyzed with the FACScan analyzer (Becton Dickinson, Milan, Italy).

### Immunohistochemical studies

Ninety-three tumor specimens from NT patients at diagnosis were tested. Patients stage was determined according to the International Staging System for Neuroblastoma [4]. This investigation was performed after approval by a local institution review board. The immunohistochemical study was carried out by means of the Dako HercepTest (K5204; Dako, Copenhagen, Denmark) according to the manufacturer's instructions. The test is a two-step immunohistotochemical staining method for routinely processed, paraffin-embedded specimens. Paraffin tissue blocks from 93 NT were sectioned at 3 μm, deparaffined in xylene, then re-hydrated through alcohol to distilled water. Two sections for each tumor were used. Antigen retrieval was performed in a steam bath at 95–99°C for 40 min. Endogenous peroxidase was inhibited by incubating the slides with peroxidase blocking reagent (3% hydrogen peroxide containing 15 mmol/l sodium azide), provided with the kit. Then, one slide was incubated with the rabbit anti-human HER2/neu protein polyclonal antibody and the other slide with the negative control reagent (an immunoglobulin fraction of normal rabbit serum at the same concentration as the antibody to HER2/neu) for 30 min. A vizualisation reagent was then used, consisting of both secondary goat anti-rabbit immunoglobulin molecules and horseradish peroxidase molecules linked to a common dextran polymer. DAB chromogen (3.3'-diaminobenzidine chromogen solution) was subsequently added: its enzymatic conversion results in the formation of a visible reaction product at the antigen sites. The specimens were counterstained in hematoxylin and then coverslipped. The kit includes positive control slides, each containing sections of three formalin-fixed, paraffin-embedded breast carcinoma cell lines representing different levels of HER2/neu protein expression.

The results were interpreted blindly by four pathologists using a light microscope according to the scoring system established by the manufacturer. HercepTest is interpreted as negative (0 and 1+ staining intensity: the former indicating no staining or membrane staining in less than 10% of the cells; the latter faint and partial membrane staining in more than 10% of the cells), weakly positive (2+ staining intensity: indicating weak-to-moderate complete membrane staining in more than 10% of the cells) and strongly positive (3+ staining intensity: indicating strong and complete membrane staining in more than 10% of the cells). Thus, only scores 2+ and 3+ indicate HER2/neu over-expression. Intra- and inter-observer reproducibility was excellent by statistical agreement *k*-estimation. Non-specific staining in the negative control occurred only in two cases, which were not included in the study.

### Statistical analysis

Statistical agreement *k*, uni and multivariate statistical analyses were performed with the SAS v.8 system and the S-plus 2000 software programs.

## Results

### HER2/neu expression in human and murine NB cell lines

Fourteen human and 2 murine NB cell lines were stained with anti-HER2/neu mAb and analyzed by flow cytometry. Only the ACN and GI-ME-N cell lines contained about 30% positive cells with a low relative mean fluorescence intensity (Table 1). All the other human and the two murine cell lines gave a percentage of positive cells and a mean fluorescence intensity superimposable on that obtained with an isotype-matched irrelevant antibody. To investigate intracellular HER2/

**Table 1** Expression of HER2/neu in human and murine NB cell lines

| Cell lines | Anti HER2/neu | | Isotype-matched irrelevant mAb | |
|---|---|---|---|---|
| | % of positive cells | Relative mean fluorescence intensity | % of positive cells | Relative mean fluorescence intensity |
| ACN | 27.9 | 8.5 | 0.3 | 2.7 |
| GI-ME-N | 33.8 | 7.7 | 0.2 | 3.2 |
| GI-LI-N | 6.9 | 4.7 | 3.0 | 3.7 |
| GI-CA-N | 2.5 | 3.6 | 1.4 | 2.9 |
| SK-N-BE 2(c) | 2.4 | 4.4 | 2.1 | 4.3 |
| IMR-32 | 0.7 | 6.7 | 0,8 | 2,89 |
| IMR-5 | 0.5 | 4.1 | 1.5 | 4.2 |
| SK-N-FI | 0.4 | 2.6 | 1.0 | 3.5 |
| SK-N-AS | 1.5 | 3.2 | 0.4 | 3.1 |
| SK-N-SH | 1.9 | 3.3 | 1.8 | 3.2 |
| SH-SY-5Y | 0.6 | 3.4 | 0.8 | 3.3 |
| LAN-5 | 1.7 | 2.8 | 0.8 | 2.5 |
| LAN-1 | 1.3 | 4.6 | 1.0 | 4.5 |
| Neuro2a | 0.3 | 3.2 | 0.1 | 3.3 |
| NXS2 | 1.0 | 3.2 | 0.6 | 3.2 |

neu protein expression in the latter cells, western blot analysis was performed on a selected number of HER2/neu-negative cell lines. The western blot results excluded the presence of HER2/neu protein retained intracellularly (data not shown), in agreement with the findings obtained by flow cytometry.

### HER2/neu expression in human NT

Ninety-three tumor specimens from NT patients at diagnosis were analyzed for HER2/neu expression by the FDA-approved DAKO HercepTest [12]. The distribution of specimens with respect to patients' stage is shown in Table 2. The results of the experiments are summarized in Table 3, and representative results are shown in Fig. 1. Most of the tumor specimens had a score of 0, and six additional samples had a score of 1, which is considered negative. Eleven samples had a score of 2 and only two samples a score of 3. Thus, 13 of the 93 tumor specimens (14%) were considered positive.

**Table 2** Distribution of tumor specimens with respect to patients' stage

| Stage | *N* |
|---|---|
| 1 | 1 |
| 2a | 20 |
| 2b | 23 |
| 3 | 14 |
| 4 | 34 |
| 4 s | 1 |
| Total | 93 |

**Table 3** HercepTest scores obtained in the 93 human NT tumors

| Score | *N* | % |
|---|---|---|
| 0 | 74 | 79.6 |
| 1 | 6 | 6.4 |
| 2 | 11 | 11.8 |
| 3 | 2 | 2.1 |
| Total | 93 | |

### Relationship between HER2/neu overexpression and patients' clinical features

No significant association was found between HER2/neu over-expression and stage, age, sex, ploidy, histological type and subtype. Moreover, log rank test indicated that overall and event-free survival were not significantly different in HER2/neu-positive and -negative patients.

## Discussion

The results obtained with both cell lines and tumor specimens clearly indicated that HER2/neu over-expression is rare in NT and unrelated to any clinical feature. Moreover, the overall survival was not significantly different between HER2/neu-negative and -positive patients. A previous study on 27 NT specimens [15] suggested that HER2/neu over-expression was a negative prognostic factor, in analogy to that reported in several other types of malignancies [5, 7, 8, 10, 13, 20, 23, 24, 25], but no scoring of HER2/neu expression was given. Conceivably, the large number of tumor samples tested in this study and the quantitative technique used for detection of HER2/neu over-expression have nullified the prognostic relevance of this parameter in NT. We did not investigate whether HER2/neu positivity was accompanied by HER2/neu gene amplification since the aim of our study was to evaluate the feasibility of HER2/neu-based immunotherapy in NT patients. This approach requires a consistent expression of the protein on the tumor cell membrane; furthermore it has been

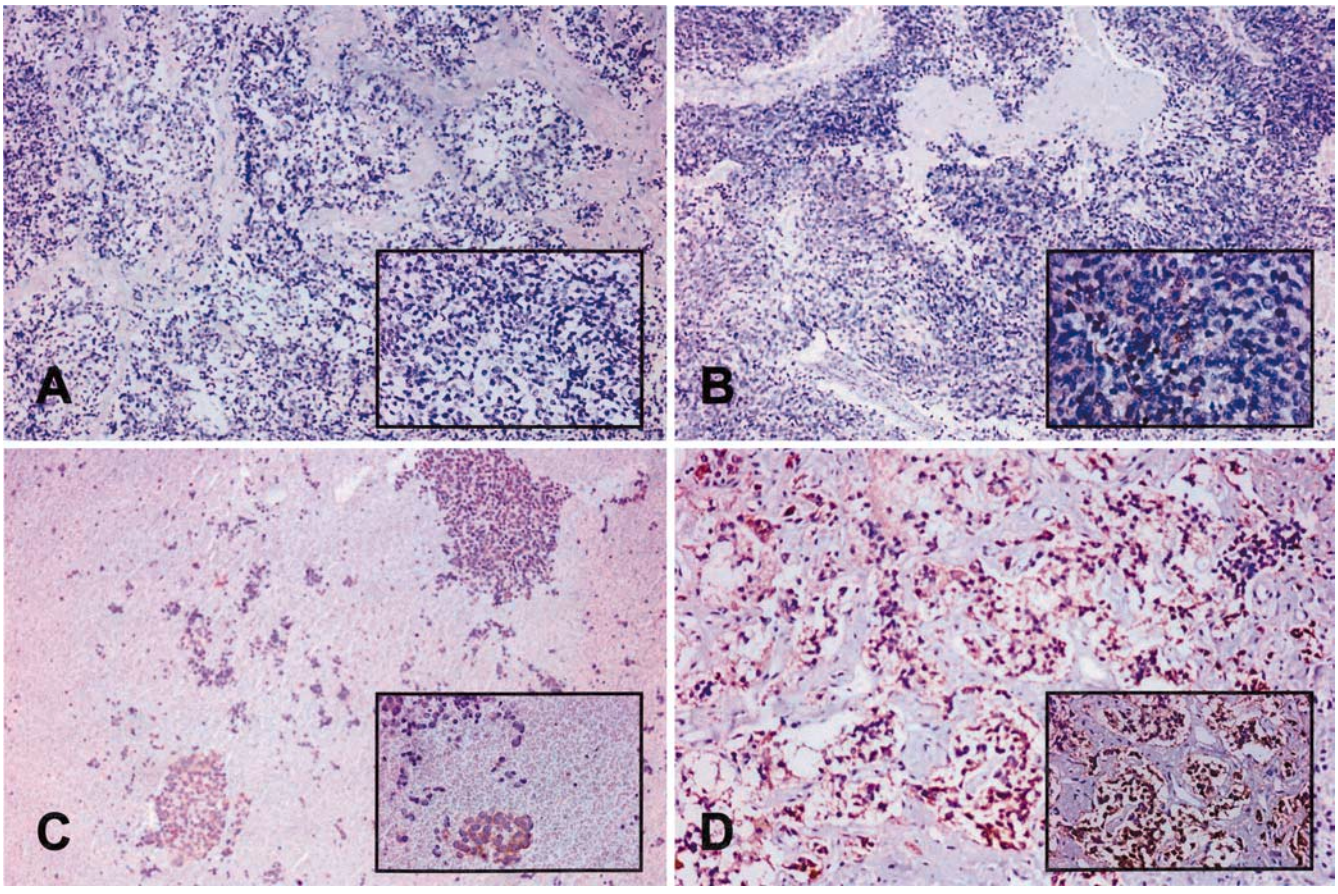


**Fig. 1A–D** HercepTest results for human NT specimens. A representative score of 0 (**A**), 1 (**B**), 2 (**C**) and 3 (**D**) obtained in NT are shown at 10x magnification. Inserts are at 40x magnification

demonstrated that HER2/neu gene amplification is more frequent than over-expression [21].

Our results do not support HER2/neu-based innovative therapy in NT patients and further investigation is needed to identify novel NB-associated antigens. Recently, several reports have demonstrated that HER2/neu over-expression is a rare event in some types of cancer such as melanoma [6], non-Hodgkin's and Hodgkin's lymphomas [2] and hepatocarcinoma [11]. In addition, HER2/neu over-expression has not been found to represent a negative prognostic factor in colorectal cancer [18] and osteosarcoma [1]. In principle, HER2/neu over-expression should be restricted to epithelium-derived tumors, thus the few cases of HER2/neu positivity observed in NB cell lines and NT might be related to a particular developmental stage of neurocrest-derived cells.

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