Cell Surface Expression of Epidermal Growth Factor Receptor and Her-2 with Nuclear Expression of Her-4 in Primary Osteosarcoma

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ABSTRACT

There is controversy over the role of Her-2 in osteosarcoma, with some investigators reporting association between expression and adverse outcome, whereas others point to the lack of gene amplification and membranous expression by immunohistochemistry (IHC) as inconsistent with biological significance. Her-2 normally requires pairing with epidermal growth factor receptor (EGFR), Her-3, or Her-4, but these have been less well studied in osteosarcoma. We evaluated the expression of each of these receptors in osteosarcoma and their potential to contribute to pathogenesis by examining a panel of low-passage primary osteosarcoma cell lines, comparing these with archival tumor specimens. Her-2 immunoreactivity was seen frequently in the diffuse staining pattern described previously. We observed EGFR in all samples by IHC. Her-3 expression was not observed. Her-4 expression was nuclear in distribution in all tumor samples and many cell line samples, consistent with activation and cleavage of the receptor. Quantified expression of Her-2 and EGFR mRNA by quantitative, real-time PCR in cell lines correlated with IHC for Her-2 but not for EGFR. Western blot identified full-length receptors for EGFR and Her-2 in all expected cell lines and showed Her-4 to be predominantly in the p80 form. Flow cytometry identified cell surface Her-2 and EGFR in all lines with receptor expression by IHC. We conclude that the cell surface expression of Her-2 and EGFR and the nuclear localization of the activated p80 fragment of Her-4 suggest that all three may be contributing to osteosarcoma pathogenesis. Therapy directed against this family of receptors may be beneficial for patients with osteosarcoma.

INTRODUCTION

Osteosarcoma is the most common solid tumor in teenagers and the third most common solid tumor in children, accounting for over 900 new diagnoses in the United States each year (1–3). Despite the use of adjuvant chemotherapy, 5-year survival has reached a plateau of approximately 70%, and this figure has not changed significantly in almost 20 years (1, 4–7). Patients with metastatic disease or relapse have a much worse outcome, and extrapulmonary metastatic disease is almost uniformly fatal (5, 8).

There has been tremendous interest in identifying markers of prognostic and therapeutic significance. One possible target in solid tumors is the erbB family of type I protein receptor tyrosine kinases (RTKs), consisting of epidermal growth factor receptor (EGFR), Her-2, Her-3, and Her-4. These cell surface molecular targets make homodimers and heterodimers (9–11) to form growth factor receptors that can mediate more rapid growth in malignant cells (12) and participate in osteosarcoma pathogenesis. To this end, we examined a panel of low-passage, primary osteosarcoma cell lines, as well as the established osteosarcoma lines Saos-2 and SJSA, for expression of all members, each of which does have ligands (16, 17). Other erbB family members preferentially pair with Her-2 when both are expressed (18, 19). Immunohistochemical stains of Her-2 expression in Her-2 overexpressing epithelial tumors showed strong antigen staining along the edges of tumor cells, consistent with membrane staining (14). This phenotype is often associated with gene amplification within malignant clones. Administration of trastuzumab, a monoclonal antibody (MAb) specific for Her-2, has led to improved outcomes for breast cancer patients with elevated Her-2 expression, especially when combined with multiagent chemotherapy (20). In addition, several small molecules have been or are being developed that specifically inhibit signaling by Her-2, EGFR, or the entire family of receptors (21).

Her-2 expression in osteosarcoma is an area of controversy. Three groups have reported an association between low-level Her-2 expression observed in initial biopsy samples and worse outcomes, including more frequent metastases and relapses and a reduced survival (22–25). Other groups did not see this association (26–29), and one group reported an association between Her-2 expression and increased overall survival (30). Another report showed that expression was not uniform between primary and metastatic tumors, including instances in which metastatic tumors lost Her-2 expression that had been observed in the primary biopsy (26). Her-2 gene amplification in osteosarcoma has not been routinely observed (27), except in a single study using fluorescence in situ hybridization analysis (25). When the protein is detected by immunohistochemistry (IHC), the pattern of staining is faint and diffuse, which pathologists associate with cytoplasmic rather than cell surface or membrane expression. It has been unclear how Her-2 expression could affect osteosarcoma biology unless the receptor is also expressed on the cell surface.

The cellular localization of erbB family members can be indicative of their phosphorylation and activation status. Some established osteosarcoma lines are known to express EGFR (31), the regulation of which includes ligand-induced internalization into endosomes (32), from which the receptor continues to signal until degraded (33). For Her-4, ligand binding and phosphorylation leads to proteolysis in two steps (34–36), resulting in a M, 80,000 fragment that retains kinase activity and traffics to the nucleus (35, 37). The pattern of expression of other erbB family members could aid the interpretation of Her-2 expression by osteosarcoma and might suggest whether RTKs are active in this disease.

We wished to determine the expression pattern for the erbB family of RTKs, attempting to either confirm or refute their ability to participate in osteosarcoma pathogenesis. To this end, we examined a panel of low-passage, primary osteosarcoma cell lines, as well as the established osteosarcoma lines Saos-2 and SJSA, for expression of all four RTKs by IHC and Western blot. The immunohistochemical expression was compared with archival tumor specimens to validate the use of these lines as models for osteosarcoma. The expression of Her-2 and EGFR was quantified at the RNA level using quantitative, real-time PCR (Q-PCR), and cell surface Her-2 and EGFR were assayed using flow cytometry. Collectively, these studies define the expression and localization of these RTKs and their ability to participate in pathogenesis of osteosarcoma.

Received 10/1/03; revised 1/15/04; accepted 1/19/04.

Grant support: NIH Grant 1 F32 CA097907-01 (D. P. M. Hughes), The Evan Shapiro Fund to Combat Children’s Cancer (D. P. M. Hughes and D. G. Thomas), and NIH Grant ROI/CA92488-01 (K. T. McDonagh).

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Note: D. G. Thomas is a Fellow of the Walther Cancer Institute.

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EXPRESSION OF erbB FAMILY MEMBERS IN PRIMARY OSTEOSARCOMA

MATERIALS AND METHODS

Cell Lines. All human tumor cell lines were obtained/derived with the approval of the Institutional Review Board of the University of Michigan Medical Center. Cells were cultured in DMEM supplemented with 10% FCS, penicillin (100 units/ml), streptomycin (100 μg/ml), and t-glutamine (292 μg/ml; Gibco/Invitrogen, Grand Island, NY) and 1% insulin/transferrin/ selenium (Gibco). Primary osteosarcoma cell lines WOL, COL, JOL, and KOL were derived from patients treated at the University of Michigan. Lines OS-179 and OS-187 were the kind gift of Dr. Richard Gorlick, Memorial Sloan Kettering Cancer Center (New York, NY). The stable cell lines Saos-2 (38) and SJSA (39) are available from American Type Culture Collection, as are control cell lines SKOV-3 (40), MCF-7 (41), and A431 (42).

Immunohistochemical staining was performed on the DAKO Autostainer (Dako, Carpinteria, CA) using DAKO LSAB+ and 3′,3′-diaminobenzidine as the chromogen. Deparaffinized sections of formalin-fixed tissue lines and control samples. The relative abundance of each species of cDNA per 10,000 copies of GAPDH, is shown. Legend: IHC, immunohistochemistry; Q-PCR, quantitative, real-time-PCR; WB, Western blot; FACS, fluorescence-activated cell sorting or flow cytometry; EGFR, epidermal growth factor receptor; M or Memb, immunoactivity pattern consistent with membranous expression; D or Diffuse, immunoactivity pattern diffuse, consistent with cytoplasmic expression; N or Nuclear, immunoactivity predominantly observed in the nuclear region, consistent with nuclear expression. ND, not done. Values for quantitative PCR and fluorescence-activated cell sorting were obtained as described in Table 1 and Fig. 4, respectively. Relative expression levels for immunohistochemistry and Western blot are described on a linear scale from 0 to 4.

Flow Cytometry. Tumor cell monolayers were mobilized with enzyme-free cell dissociation buffer (In Vitrogen) and resuspended at a density of 2 × 10⁷ cells/ml. Cells (10⁶) were stained with phycoerythrin-conjugated anti-EGFR (clone EGFR-1; Becton Dickinson, San Jose, CA), phycoerythrin-conjugated anti-Her-2 (clone Neu 24.7; Becton Dickinson), or phycoerythrin-conjugated isotype control (mouse IgG1/k; Pharmingen, San Diego, CA) and analyzed on an EPICS XL flow cytometer using XL System II software (Beckman-Coulter). Living cells were identified by forward scatter/side scatter gating. To quantify receptor expression, standardized antibody-binding beads (Quantum Simply Cellular; Bangs Laboratories, Fishers, IN) were stained in parallel with tumor cells. These beads bind antibody at precisely defined numbers of MAb molecules/bead (five different densities), allowing comparison via regression analysis between fluorescence intensity and antibody number. Because MAb specific for EGFR or Her-2 were used at saturating concentrations, one and only one MAb was attached to each cell surface receptor. The background value of isotype control-stained cells was subtracted from the calculated density of Her-2 and EGFR to obtain the actual number of molecules/cell.

RESULTS

Detection of erbB Family Member Expression in Osteosarcoma by IHC. To determine the potential for EGFR, Her-2, Her-3, and Her-4 to participate in osteosarcoma pathogenesis, we cultured low-passage, primary osteosarcoma cell lines in vitro and made paraffin-embedded cell blocks of these for examination by IHC. As a comparison, we also studied the established cell lines Saos-2 (38) and SJSA (39), as well as two control lines, MCF-7 (41), a breast cancer line that does not overexpress Her-2, and SKOV-3 (40), an ovarian carcinoma line that overexpresses Her-2 (45). Cell sections were stained with H&E, EGFR, Her-2, Her-3, or Her-4 and examined by light microscopy (Fig. 1). Semiquantitative measures of expression, with a description of the staining pattern, are included in summary Table 1. The primary cell lines demonstrated a range of Her-2 expression, from line COL, which showed no Her-2, to cell line OS-187, which demonstrated strong immunoreactivity, although less intense than that of SKOV-3. In the majority of osteosarcoma cell lines, including Saos-2 and SJSA, Her-2 expression was faint and diffusely present throughout the cell, a pattern consistent with cytoplasmic rather than membrane expression. All osteosarcoma cell lines were

Table 1: Summary of erbB family expression in osteosarcoma cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>EGFR IHC</th>
<th>EGFR Q-PCR</th>
<th>EGFR WB</th>
<th>EGFR FACS</th>
<th>Her-2 IHC</th>
<th>Her-2 Q-PCR</th>
<th>Her-2 WB</th>
<th>Her-2 FACS</th>
<th>Her-4 IHC</th>
<th>Her-4 WB</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL</td>
<td>4 Membr</td>
<td>164</td>
<td>+++</td>
<td>38</td>
<td>-</td>
<td>3 + D + N</td>
<td>3 + D + N</td>
<td>+</td>
<td>+</td>
<td>+ + + +</td>
</tr>
<tr>
<td>JOL</td>
<td>4 Diffuse</td>
<td>610</td>
<td>+ + N</td>
<td>1 + Diffuse</td>
<td>130</td>
<td>+</td>
<td>23</td>
<td>2 + Diffuse</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KOL</td>
<td>ND</td>
<td>2241</td>
<td>+</td>
<td>ND</td>
<td>905</td>
<td>+</td>
<td>23</td>
<td>ND</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>WOL</td>
<td>4 + M + D</td>
<td>1940</td>
<td>+ + 40</td>
<td>Trace Diffuse</td>
<td>16,841</td>
<td>+</td>
<td>35</td>
<td>4 + Nuclear</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>OS-179</td>
<td>4 Diffuse</td>
<td>3930</td>
<td>+</td>
<td>Trace Diffuse</td>
<td>1170</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>OS-187</td>
<td>4 Membr</td>
<td>712</td>
<td>+++</td>
<td>62</td>
<td>2 + M + D</td>
<td>26,032</td>
<td>+ + + +</td>
<td>188</td>
<td>4 + D + N</td>
<td>++</td>
</tr>
<tr>
<td>Saos-2</td>
<td>2 Membr</td>
<td>ND</td>
<td>+ 13</td>
<td>1 + Diffuse</td>
<td>ND</td>
<td>+</td>
<td>49</td>
<td>3 + D + N</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SJSA</td>
<td>4 + M + D</td>
<td>ND</td>
<td>ND 49</td>
<td>Trace Diffuse</td>
<td>ND</td>
<td>ND</td>
<td>33</td>
<td>2 + D + N</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Trace Membr</td>
<td>10</td>
<td>+ 8</td>
<td>1 Membr</td>
<td>12,208</td>
<td>+</td>
<td>71</td>
<td>4 + Nuclear</td>
<td>++ + + +</td>
<td>++</td>
</tr>
<tr>
<td>SKOV-3</td>
<td>2 Membr</td>
<td>4497</td>
<td>? 41</td>
<td>4 + M + D</td>
<td>53,214</td>
<td>+ + + +</td>
<td>1,146</td>
<td>2 + D + N</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
immunopositive for EGFR in a pattern consistent with membranous expression. No Her-3 expression was observed. The expression of Her-4 was diffusely present throughout the cell, except for cell line WOL and scattered cells in other samples, which appeared to have nuclear localization of antigen.

It was important to demonstrate that these primary osteosarcoma cell lines could serve as an accurate model for the EGFR family member expression observed for osteosarcoma in patients. To validate the expression in these cells, we examined archival osteosarcoma tumors from primary biopsies, tumors resected after neoadjuvant chemotherapy, and recurrent, metastatic pulmonary nodules for the expression of EGFR, Her-2, Her-3, and Her-4. This panel was intended to demonstrate the range of expression seen in human disease but did not have a sufficient quantity of tumors to permit correlation between expression and outcome. Her-2 in this panel demonstrated varied expression, with no Her-2 in some samples, low-level diffuse staining in the majority of cells in other samples, and focal, higher level cytoplasmic expression in a minority of cells in other tumors. Most samples had uniform, strong immunopositivity for EGFR in a diffuse pattern. Her-3 was negative in all tumors. Her-4 immunoreactivity showed nuclear localization in most archival tumor specimens. Representative tumors are shown in Fig. 2. Semiquantitative measures of expression, with a description of the staining pattern, are included in summary Table 1. The similarity in EGFR family member expression between the primary osteosarcoma cell lines and archival pathological specimens validated the use of the primary osteosarcoma cells as a model for EGFR family member functioning 

Detection of erbB Family Member mRNA in Osteosarcoma Cell Lines by Q-PCR. We wished to obtain further evidence for the presence of erbB family members in primary osteosarcoma cell lines. mRNA was extracted from primary osteosarcoma cell lines grown in parallel with those examined by IHC, as well as from the control lines MCF-7 and SKOV-3, and cDNA was made by reverse transcription. The cDNA was assayed by Q-PCR for the presence of Her-2 and EGFR expression, as well as for expression of the housekeeping gene GAPDH. The amount of cDNA (in copies/μg RNA) was calculated for each of the three genes, and the number of copies of Her-2 and/or EGFR per 10,000 GAPDH copies was calculated (Table 1). Her-2
cDNA was detected from all cell lines, and the amount of message approximated the intensity of staining in IHC. EGFR cDNA was detected from all cell lines tested, although no relationship between intensity of staining in IHC and number of cDNA copies was identified.

Detection of erbB Family Members in Osteosarcoma by Western Blot. We demonstrated that primary osteosarcoma cell lines express EGFR and Her-2 at the mRNA level and show staining for these and for Her-4 by IHC. We wished to know whether the translation of this message leads to full-length RKT expression in primary osteosarcoma cell lines. To assess the expression of full-length proteins of the erbB family, we made protein lysates from parallel cultures of the same cells assayed in Fig. 1. These lysates were separated by SDS-PAGE and examined by Western blot for EGFR, Her-2, Her-3, and Her-4 (Fig. 3). Semiquantitative measures of Western blot intensity are included in summary Table 1. To avoid overexposure during Her-2 detection, only 5% of the protein was loaded from the SKOV-3 sample (which is known to overexpress Her-2) as was analyzed for the other samples.

Her-2 was detectible (in decreasing intensity) from cell lines OS-187, OS-179, KOL, WOL, Saos-2, and JOL and was the same size as Her-2 expressed in SKOV-3 and MCF-7. Her-2 expression was below the threshold of detection by this method for COL. p170-EGFR was seen at the correct size for all primary lines. Her-3 was not detected in primary osteosarcoma lines. Her-4 signal was detected in COL, OS-187, WOL, Saos-2, and MCF-7; faintly detected in OS-179, JOL, and KOL; and observed at a molecular weight of $M_r$ 80,000 for all samples. Thus, primary osteosarcoma cell lines express full-length proteins for Her-2 and EGFR and contain the p80 fragment of Her-4.
Detection of Cell Surface Her-2 and EGFR Expression by Flow Cytometry. The pattern of antigen detected by IHC of osteosarcoma tumor samples for Her-2, Her-4, and, to a lesser extent, EGFR, is usually interpreted by pathologists as signifying cytoplasmic rather than membrane expression. For a RTK to bind ligand and signal, it is assumed that the receptor must be expressed at the cell surface, at least transiently. We wished to determine whether the erbB family members expressed in osteosarcoma cells were present on the cell surface, establishing their ability to participate in ligand binding and signaling events.

To detect cell surface expression of Her-2 and EGFR in primary osteosarcoma cell lines, we used flow cytometry. Viable osteosarcoma cells and control samples were stained with phycoerythrin-conjugated anti-Her-2 MAb, anti-EGFR MAb, or isotype control antibody. A431 cells, an epithelial carcinoma line known to overexpress EGFR, were included as a positive control for EGFR staining. Single overlay histograms are shown. The number of receptor molecules/cell, calculated using standardized reference beads and expressed in thousands, is shown in the top right corner of each panel.

**DISCUSSION**

Regulation of Expression of the erbB Family of Receptors. The trafficking and signaling of the RTKs of the erbB family are complex, with different regulatory mechanisms described for various receptors. Because clear evidence for expression of three of the four by primary osteosarcoma lines was observed, it is helpful to briefly review their described behaviors. The same mechanisms may be active in osteosarcoma and may help explain the varied observations made, both by our group and others, of Her-2/EGFR expression in osteosarcoma.

The extracellular region, intracellular kinase domain, and COOH-terminal domain responsible for autophosphorylation of RTKs are well described [reviewed by Jorissen et al. (47)]. On ligand binding, homodimers and heterodimers of these RTKs undergo autophosphorylation (48), leading to second messenger generation through the RAS/mitogen-activated protein kinase (49), Shc (50), Src (51), phospholipase C-γ (52), phospholipase D (53), and/or signal transducers and activators of transcription (54, 55) pathways, and possibly others. EGFR, upon binding ligand and becoming phosphorylated, undergoes rapid internalization in clathrin-coated pits and endosomes, perhaps together with its pairing partners such as Her-2, from which it continues to signal for up to an hour before being degraded (32). Thus ligand binding of EGFR, with surface down-regulation and degradation, can cause Her-2 degradation as well (33).

An additional level of regulation by proteases had been described for Her-4. Binding of neuregulin by a Her-4-containing homodimer or heterodimer induces cleavage of the extracellular domain from Her-4 by ADAM17 (also called TACE), a metalloproteinase (34). This cleavage releases a M, 120,000 fragment consisting of the entire extracellular region, whereas a M, 80,000 fragment is retained bound to the membrane. After ADAM17 cleavage either at the surface or within internalized smooth vesicles, the transmembrane domain of Her-4 can be cleaved by γ-secretase (also called PS-1), which releases the entire intracellular domain into the cytoplasm (35, 36). This fragment retains tyrosine kinase activity and can be translocated to the nucleus. Its substrates there are unknown. Thus the regulation of the erbB family includes internalization of active kinases.

**Her-2 Expression in Osteosarcoma.** There has been great controversy over the expression of Her-2 in osteosarcoma and its potential role in pathogenesis. We establish here that primary osteosarcoma cell lines do express the receptor, albeit at much lower levels than that observed in some breast or ovarian cancers. The cell surface localization for the receptor was found even in those cell lines that appeared to have a “cytoplasmic” pattern of Her-2 staining by IHC. This low level of expression has been associated with adverse outcome in some studies (22–25), but not others (26–30).

One reason for this controversy is the substantial difference in receptor levels expressed by osteosarcoma compared with that of the epithelial malignancies in which the function of EGFR and Her-2 was first described. We demonstrate that primary osteosarcoma lines can express both Her-2 and EGFR on the cell surface. We also show that typical Her-2" osteosarcoma lines such as WOL have about 50-fold less cell surface Her-2 than a typical ovarian cancer line, SKOV-3. There was also about a 20-fold lower level of cell surface expression of EGFR than an EGFR overexpressing epithelial carcinoma line. An
inherent assumption in many investigations of RTK function in osteosarcoma was that the gene and protein would act in the same manner that they do in breast cancer, ovarian cancer, or non-small cell lung cancer, where adverse outcome is associated with overexpression, not the presence of the receptor per se. Thus a wealth of studies have pointed to the lack of gene amplification for Her-2 in osteosarcoma, the failure of immunohistochemical staining to demonstrate a membranous pattern for Her-2, and the scant staining observed as evidence that the receptor could not be contributing to osteosarcoma biology.

Whereas such an approach may make intuitive sense, the RTKs are clearly able to influence cell growth and tissue development when expressed at lower levels. The animal models for this family of receptors, including knockout mice (56, 57), have shown their essential functions when expressed at low density in normal tissues. There is sufficient evidence to admit the possibility that Her-2 signaling may be important to the pathogenesis for some osteosarcoma, and the present work demonstrates cell surface localization of Her-2 that may account for such signaling. Additional studies will be needed to assess which Her-2 signaling pathways may be functioning in osteosarcoma.

**EGFR Expression in Osteosarcoma.** The EGFR expression in osteosarcoma at a minimum would provide a pairing partner for any Her-2, facilitating signaling from both members of the family. Previous studies found evidence for expression of both EGF and EGFR by stable osteosarcoma cell lines (58–61) and in archival specimens (62), although this expression has received little attention. Discrepancies were seen in our studies between EGFR measured by one technique compared with others. All of our osteosarcoma lines express readily detectable EGFR by Q-PCR and IHC, and the antigen is seen predominantly in a membranous pattern, suggesting cell surface expression. By contrast, EGFR expression in archival specimens was observed predominantly in a cytoplasmic pattern, consistent with ligand binding, activation, and internalization in vivo. Flow cytometry of osteosarcoma lines also demonstrated cell surface EGFR expression, although the density of expression at the surface (in molecules/cell) was lower than one would have anticipated based on the immunohistochemical staining and the Western blot analysis. For example, COL has much more EGFR than either WOL or OS-187 by Western blot analysis but has lower cell surface expression by flow cytometry. It is clear from both the Western blot and IHC that the receptor is present in most cells but may be partially protected from staining detectible by fluorescence-activated cell sorting.

One explanation for the discrepancy between flow cytometric analysis and the IHC or Western analysis would be if much of the EGFR in osteosarcoma is contained in endocytosed vesicles. Endocytosis would be expected if EGFR were binding ligand, either growth factors in the culture medium or EGF synthesized and secreted by the osteosarcoma cells themselves. The culture medium seems an unlikely source of a ligand inducing internalization because high-density expression of EGFR was seen on the surface of A431 cells grown in the same medium. Internalized receptor would also explain the diffuse staining pattern observed for archival tumors in IHC. In systems where EGFR and Her-2 are expressed at similar densities, the EGFR promotes trafficking of Her-2 contained in heterodimers into coated pits, and both receptors are phosphorylated and activated (33). Thus, the pattern of expression for EGFR and Her-2 suggests that both have the potential to affect osteosarcoma behavior. Because EGFR is internalized at a much higher rate when the receptor is phosphorylated (33), our observation of RTK internalization in osteosarcoma raises questions about the expected efficacy of immune-based therapies targeting either of these receptors with MAbs such as trastuzumab because much of the receptor may be shielded in internalized vesicles.

**Her-3 and Her-4 Expression in OS.** Significant Her-3 expression was not observed by any of the methods used, except in the control line MCF-7. Thus, it is unlikely that Her-3 makes a significant contribution to osteosarcoma pathogenesis or outcome. The lack of good antibodies to detect Her-3 or Her-4 by flow cytometry makes it difficult to further assess the expression and trafficking of these receptors as we have done for EGFR and Her-2.

The pattern of expression seen for Her-4 by IHC was generally concordant with the protein expression measured by Western blot. Her-4 staining in archival osteosarcoma tumor samples was almost exclusively nuclear in distribution, suggesting an activated state. A subset of the WOL, SJSA, and COL cells also appear by IHC to have nuclear localization of Her-4 antigen, which would suggest generation of the p80 fragment by γ-secretase or a related enzyme (35, 36). It is unclear what happens to the Her-2 bound into heterodimers with activated Her-4 when the Her-4 is cleaved by γ-secretase. Western blot confirmed that Her-4 is present in the p80 form in these osteosarcoma cells. Because this fragment retains its kinase activity and is generated from phosphorylated, active receptors, this observation would indicate that the Her-4 observed in osteosarcoma is activated and signaling.

We have shown that EGFR, Her-2, and Her-4 are expressed in primary, low-passage osteosarcoma cell lines and that at least the first two are present on the cell surface and as intact, full-length proteins within the cell. The pattern of expression for EGFR and Her-4 suggests that both are activated and signaling in most osteosarcoma cell lines. Her-2 expression is varied among primary osteosarcoma lines but is present on the surface of primary osteosarcoma cells in which it is observed by IHC. This expression, together with the presence of activated pairing partners for Her-2, provides a biological foundation for the clinical evidence that expression of Her-2 is associated with adverse outcomes in osteosarcoma. Alternatively, it may be that expression of any of the erbB family members by osteosarcoma confers a worse prognosis. Those studies remain to be performed. In either case, the frequency with which members of this family are observed in osteosarcoma suggests that these patients might benefit from pharmacotherapy targeting RTK signaling, a possibility we will investigate in additional studies.

**ACKNOWLEDGMENTS**

We thank Michelle Lizyness and Angela Smith for technical assistance and Dr. Stephen Ehier for critical review of the manuscript. Cell lines OS-179 and OS-187 were the kind gift of Dr. Richard Gorlick.

**REFERENCES**

9. Yarden Y, Schlessinger J. Epidermal growth factor induces rapid, reversible aggre-
gation of the purified epidermal growth factor receptor. Biochemistry 1987;26:1431–
51.
10. Yarden Y, Schlessinger J. Self-phosphorylation of epidermal growth factor receptor: evidence for a model of intermolecular allosteric activation. Biochemistry 1987;26:
1434–42.
factor receptor-kinase complex from shed plasma membrane vesicles. J Biol Chem
12. Neve R, Holbro T, Hynes N. Distinct roles for phosphoinositide 3-kinase, mitogen-
activated protein kinase and p38 MAPK in mediating cell cycle progression of breast
tion of epidermal growth factor receptor and transactivation by insulin-like growth
versus survival with amplification of the HER-2/neu oncogene. Science (Wash. DC)
1987;235:177–82.
carcinomas may function solely as a shared coreceptor for multiple stroma-derived
17. Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor
interactions determines signal transduction by Neu differentation factor/neuregulin
19. Graus-Porta D, Beerli RR, Daly JM, Hynes NE. ErbB-2 expression is correlated with increased
expression of cytokinin-kinase among various histologic subtypes and grades of osteosar-
trastuzumab (Herceptin) in patients with HER2/neu-overexpressing metastatic breast
encoding a p53-associated protein in human sarcomas. Nature (Lond.) 1992;358:
103.
signaling through the stromal epidermal growth factor receptor kinase. J Biol Chem
and survival with amplification of the HER-2/neu oncogene. Science (Wash. DC)
1987;235:177–82.
29. Li Y, Serrano M, Lin AW, et al. Distinct roles for phosphoinositide 3-kinase, mitogen-
activated protein kinase and p38 MAPK in mediating cell cycle progression of breast
30. Akatsuka T, Wada T, Kokai Y, et al. ErbB expression is correlated with increased
expression of cytokinin-kinase among various histologic subtypes and grades of osteosar-
encoding a p53-associated protein in human sarcomas. Nature (Lond.) 1992;358:
103.
32. Shawver L, Mann E, Elliger S, Dugger T, Arteaga C. Ligand-like effects induced by
anti-erbB-2 antibodies do not correlate with and are not required for growth inhibition
33. Carlin K, Knowles B. Biosynthesis of the epidermal growth factor receptor in human
interactions determines signal transduction by Neu differentation factor/neuregulin
35. Moriki T, Maruyama H, Maruyama I. Activation of preformed EGF receptor dimers
76.
36. Slaidya R, Jensen T, Hansen HS, Froholm MA, Seedorf K. PLD2 complexes with the
EGF receptor and undergoes tyrosine phosphorylation at a single site upon agonist
38. Wiesen J, Young P, Werb Z, Cunha G. Signaling through the stromal epidermal
growth factor receptor is necessary for mammary ductal development. Development
(Camb.) 1999;126:335–44.
39. Sladek R, Jensen T, Hansen HS, Froholm MA, Seedorf K. PLD2 complexes with the
EGF receptor and undergoes tyrosine phosphorylation at a single site upon agonist
40. Hiwase J, Young P, Werb Z, Cunha G. Signaling through the stromal epidermal
growth factor receptor is necessary for mammary ductal development. Development
(Camb.) 1999;126:335–44.
41. Shawver L, Mann E, Elliger S, Dugger T, Arteaga C. Ligand-like effects induced by
anti-erbB-2 antibodies do not correlate with and are not required for growth inhibition
42. Heid C, Stevens J, Livak K, Williams P. Real time quantitative PCR. Genome Res
43. Shawver L, Mann E, Elliger S, Dugger T, Arteaga C. Ligand-like effects induced by
anti-erbB-2 antibodies do not correlate with and are not required for growth inhibition
interactions determines signal transduction by Neu differentation factor/neuregulin
45. Moriki T, Maruyama H, Maruyama I. Activation of preformed EGF receptor dimers
76.
46. Slaidya R, Jensen T, Hansen HS, Froholm MA, Seedorf K. PLD2 complexes with the
EGF receptor and undergoes tyrosine phosphorylation at a single site upon agonist
48. Wiesen J, Young P, Werb Z, Cunha G. Signaling through the stromal epidermal
growth factor receptor is necessary for mammary ductal development. Development
(Camb.) 1999;126:335–44.
49. Shawver L, Mann E, Elliger S, Dugger T, Arteaga C. Ligand-like effects induced by
anti-erbB-2 antibodies do not correlate with and are not required for growth inhibition
50. Heid C, Stevens J, Livak K, Williams P. Real time quantitative PCR. Genome Res
Correction: Cell Surface Expression of Epidermal Growth Factor Receptor and Her-2 with Nuclear Expression of Her-4 in Primary Osteosarcoma

Long after the publication of this article (1), we obtained new data showing that OS 187 and COL, 2 of the cell lines identified as osteosarcoma cells in this article, do not represent osteosarcoma.

Cell line OS 187, first obtained with 4 other cell lines as a gift from colleagues at an outside institution, was subjected to DNA fingerprinting recently, in anticipation of publishing further data. That analysis clearly showed that OS 187 was obtained from the same patient who was the source of the commonly used colon adenocarcinoma cell line HCT15 and is presumably a subline of HCT15. We subsequently were able to inject OS 187 cells orthotopically into the cecum wall of NOD/SCID/IL2γR−/− mice and have ‘primary’ colon tumors arise, with spontaneous metastasis to liver and spread to the peritoneum (data not shown), further suggesting that OS 187 is a subline of HCT15. We did a follow-up DNA fingerprint analysis on frozen cells made from the second passage of OS 187 in our laboratory in 2002, and this sample also was identical to HCT15 at all 12 loci tested, suggesting that any contamination event likely happened before the cells arriving at the University of Michigan and that all data published using the cells from our group reflects the biology of HCT15 and of colon cancer, not osteosarcoma.

COL is, as indicated in the original publication, a unique cell line we made at the University of Michigan. We recently attempted to generate an orthotopic in vivo model by injection of these cells into the tibia of NOD/SCID/IL2γR−/− mice. To our surprise, no primary tumor arose in the tibia, but tumor tissue was found in liver, bone marrow, and skin, and some animals developed retro-orbital tumors. Further testing, detailed in our recent publication (2), confirmed that COL represents a neuroblastoma cell line.

Because all experiments presented in this article included at least 2 other primary osteosarcoma cell lines and 2 additional established osteosarcoma cell lines, and because the patient-derived material is consistent with the cell line data, we believe that the results presented in this manuscript continue to reflect accurately the biology of osteosarcoma.

References


Published OnlineFirst June 21, 2013.
doi: 10.1158/0008-5472.CAN-13-1177
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Cell Surface Expression of Epidermal Growth Factor Receptor and Her-2 with Nuclear Expression of Her-4 in Primary Osteosarcoma

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*Cancer Res* 2004;64:2047-2053.

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