The expression of epidermal growth factor receptor and its downstream signaling molecules in osteosarcoma

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Abstract. The value of epidermal growth factor receptor (EGFR) and its downstream signaling molecules as prognostic factors have been studied in many tumor types. The aim of this study was to investigate whether these molecules have prognostic value in osteosarcoma. We evaluated the immunostaining of EGFR and its downstream signaling molecules, p-EGFR, Akt, Stat-3, survivin and Erk in 47 osteosarcomas. In addition, three osteosarcoma cell lines were used to evaluate EGFR expression levels and mutation status by real-time PCR and nucleotide sequence analysis. Using tissue microarray of samples from 47 paraffin-embedded osteosarcoma cases, 26, 17, 20 and 12 cases showed positive immunostaining of EGFR, Stat-3, survivin and Erk, respectively. Survivin and Erk were statistically correlated with survival (p=0.005 and p=0.002, respectively, log-rank test). Furthermore, we found that EGFR expression was correlated with Erk expression. In addition, we also observed a significant association of survivin expression with Stat-3 and Erk activation (p=0.006 and p=0.002, respectively, log-rank test). p-EGFR and Akt immunostaining were not detected in any of the cases. Two out of three osteosarcoma cell lines showed increased EGFR levels as detected by real-time PCR. One of these cell lines had a CAA to CAG mutation at exon 20 of the amplified EGFR gene, but this did not change the amino acid sequence. These results support the idea that Erk is a downstream signaling molecule of EGFR. Moreover, our data indicate that survivin and Erk could be used as prognostic factors in patients with osteosarcoma.

Introduction

The epidermal growth factor receptor (EGFR) is a member of the ErbB receptor tyrosine kinase family, which consists of four transmembrane receptors: EGFR (ErbB1/HER1), ErbB2 (HER2/neu), ErbB3 (HER3) and ErbB4 (HER4). EGFR activation affects cell division, survival, invasion, adhesion, and angiogenesis and is a factor of poor prognosis (1-3). These effects are mediated by activation of downstream signal transduction cascades that include Janus tyrosine kinase (Jak)/signal transducers and activators of transcription (Stat), phosphatidylinositol 3 kinase (PI3K)/Akt and Ras/Raf/mitogen-activated protein kinase (Erk) (4-6). In addition, survivin expression, which is downregulated by Stat, Akt, and Erk, is also a negative prognostic factor in some tumor types such as breast and lung cancer as well as head and neck cancer (7-9). Many studies have reported overexpression of EGFR in a number of common solid tumors, including non-small cell lung carcinoma, as well colon, breast, head and neck and ovarian cancers (1,10). In addition, amplification of the EGFR gene and overexpression of the EGFR protein was observed in glioblastoma (11). Therefore, several EGFR-targeted therapies are used in the clinic, including the anti-EGFR monoclonal antibody cetuximab, tyrosine kinase inhibitors (TKIs), gefitinib and erlotinib (12-16). After binding to its ligand, EGFR undergoes autophosphorylation and transduces signals through several pathways that involve Stat, Akt and survivin.

After activation by the phosphotyrosine residues of EGFR, Stat modulates apoptosis, cell proliferation and differentiation (17-19). One of the members of the Stat family, Stat-3, is expressed in various human cancers such as lung, head and neck, breast cancer types (17,20-22).

Akt is also phosphorylated and activated by EGF through PI3-kinase. Activated Akt plays a role in various cellular processes such as glucose metabolism, DNA synthesis, cell cycle progression through anti-apoptotic signaling and cell survival (23,24). In addition, hyperphosphorylation of Akt has been reported in many cases of non-small cell lung cancer (25).

Erk1/2 is activated by mitogenic agents and is a component of signal transduction cascades that regulate cell proliferation, survival and transformation (26-28). Activated Erk has been found in kidney, colon and lung cancers (27).

Survivin is a member of the inhibitor of apoptosis protein (IAP) family, which is present during fetal development but is not found in normal adult tissue (29,30). The overexpression of survivin has been observed in many kinds of cancers including colorectal, breast, lung cancer and malignant...
lymphoma and has been suggested to be a negative prognostic factor (31-34).

Osteosarcoma is a primary high-grade malignant tumor in which the neoplastic cells produce osteoid. This disease is the most common, non-hematopoietic primary malignant tumor of the bone. Despite the fact that the relationship between EGFR and downstream molecules has been evaluated in many kinds of malignant tumors, no previous studies have simultaneously examined the status of EGFR and all four downstream molecules in osteosarcoma. The aim of this study was to analyze the expression and correlation of EGFR and its downstream signaling molecules to clinicopathological data in osteosarcomas.

Materials and methods

This study was approved by the IRB of Kyung-Hee University Hospital. Forty-seven formalin-fixed, paraffin-embedded osteosarcoma case samples, which had been sampled prior to chemotherapy, were obtained for this study. The samples were collected from 1983 to 2005 in the Department of Pathology at the Kyung-Hee University Hospital and the Hallym University Hospital. All cases were evaluated by two separate investigators (D.S.I. and P.Y.K.), using hematoxylin and eosin-stained sections. Hospital records were also reviewed for each case. A 0.3-mm tissue core was taken from each sample to include on tissue microarray slides. The cores were extracted from a representative part of the tumor according to the corresponding H&E-stained slides. Immunohistochemical stains were performed on 4-μm tissue sections cut from microarray paraffin blocks using an antibody against EGFR (EGFR, Clone 31G7, Zymed, CA, USA) 1:100 and four commercial antibodies from Cell Signaling Technology (MA, USA) against the phosphorylated forms of EGFR (p-EGFR\text{\textsuperscript{Tyr1068}}, polyclonal); 1:50, p-Stat3\text{\textsuperscript{Tyr705}} (monoclonal); 1:200, p-Akt\text{\textsuperscript{Ser473}} (monoclonal); 1:100, and p-Erk\text{\textsuperscript{Tyr202/Tyr204}} (monoclonal); 3:1000. In addition, a monoclonal antibody against survivin; 1:1000 (Neomarkers, Lab vision, CA, USA) was used. A bond-max immunohistochemistry auto-stainer (Vision Biosystem, VIC, Australia) was used for immunohistochemical staining. Placental tissue served as a positive control. Positive immunohistochemistry staining was evaluated by two investigators (D.S.I. and P.Y.K.) and positive expression was defined as >10% of osteosarcoma cells with positive staining.

For real-time PCR and nucleotide sequencing, five cell lines (fibroblasts, ES97, MG63, SJSA-1 and HS3.7) were cultured in media (Gibco\textsuperscript{TM}, Invitrogen Corporation). Fibroblasts and ES97 (Ewing sarcoma) cells were used as controls, while MG63, SJSA-1 and HS3.7 served as the representative osteosarcoma cell lines in our study. RNA was isolated from cultured cells using an RNaseasy kit (Qiagen). cDNA was synthesized from the RNA by using the 1st strand cDNA kit (Roche, USA) (10X buffer 2.0 μl, 25 mM MgCl\textsubscript{2} 4.0 μl, dNTP 2.0 μl, oligo-dT primer 2.0 μl, RNasin 1.0 μl, AMV reverse transcriptase 0.8 μl, RNA 1.0 μl and sterile water) and a 9700 thermal cycler (ABI, Applied Biosystems, Foster City, USA). Real-time PCR was analyzed by using upl (Universal ProbeLibrary Probes, cat, No. 04683633001, Roche), [2X probe mixture; 10.0 μl, primer (10 pmol/μl), upl
probe; 0.2 μl, DNA (100 ng/μl); 2.0 μl and sterile water] and the Light Cycler (Roche). For mutational analysis of the EGFR kinase domain, DNA was isolated from cultured cells using Magna Pure LC (Roche Diagnostics GmbH Mannheim, Germany) followed by PCR amplification using a 9700 thermal cycler (ABI, Applied Biosystems). After PCR fragment purification, a sequencing reaction (primer; 1.0 μl, BigDye terminator v.3.1; 8.0 μl, PCR fragments; 2.0 μl and sterile water) and analysis (3100 genetic analyzer, ABI, Applied Biosystems) were performed.

Statistical analysis. Statistical analysis was carried out using SPSS software. A p-value <0.05 was considered statistically significant.

Results

In total, we analyzed 47 cases of osteosarcoma from patients ranging in age from 7 to 66 years, with a mean age of 25 years. Of these cases, 25 were male and 22 were female. The follow-up period ranged from 1 to 275 months and the mean follow-up time was 76.9 months. At the time of diagnosis, the 47 cases consisted of 20 stage I, 15 stage II, 7 stage III, and 1 stage IV tumors. In four cases, the stage had not been recorded in the hospital records. The femur was the most common tumor site (20 cases) followed by the humerus (5 cases), maxilla (4 cases), mandible (3 cases) and other sites. Of the tumors, 37 were osteoblastic, 5 were fibroblastic and 5 were chondroblastic types of osteosarcoma.

Twenty-six cases of osteosarcoma showed positive EGFR expression. Of these, 23 cases showed expression predominately in the cytoplasm and 3 cases showed expression on the membrane surface (Fig. 1A and B). Nuclear expression of Stat-3, Erk, and cytoplasmic expression of survivin were observed in 17, 12 and 20 cases, respectively (Fig. 1C, D and E). Erk and survivin expression were statistically correlated with survival (p=0.005 and p=0.002, Kaplan-Meier test, Fig. 2C and D). In addition, we observed a correlation between EGFR and Erk expression (p=0.042, Fisher's exact test). Moreover, we also observed a correlation between Stat-3 and survivin expression, as well as between Erk and survivin expression (p=0.006 and p=0.000, Fisher's exact test) (Table I). However, EGFR and Stat-3 expression were not statistically correlated with survivin (p=0.5319 and p=0.1292, Kaplan-Meier test, Fig. 2A and B). We did not observe p-EGFR and Akt expression in any of the cases. Moreover, no other correlations were found between the immunohistochemistry, staining of the molecules, and the other clinicopathological data, such as age, gender, or metastasis (Table II). In the real-time PCR results, increased EGFR and Akt levels were observed in two out of the three osteosarcoma cell lines, HS3.7 and MG63 (Fig. 3). In addition, the MG63 cell line showed increased Stat-3 levels. To investigate possible EGFR mutations, we sequenced the kinase domain from exons 18 to 21 in the cell lines. We found a point mutation which changed A to G at codon 54,031 at exon 20 (Fig. 4). However, this alteration in DNA sequence was silent i.e. it did not lead to a change in the amino acid (glutamine) at this position.

Discussion

Osteosarcoma is the most common primary malignant tumor of the bone and has an aggressive behavior. Previously, the long-term survival of patients has improved substantially as a result of neoadjuvant chemotherapy. However, despite these chemotherapy options, patients who have metastasis at the time of diagnosis or those with recurrent disease still show poor long-term survival (35). Thus a more advanced therapy for osteosarcoma is needed to achieve improved survival rates. For the same reason, previous studies have evaluated EGFR signaling and its downstream signaling molecules in many types of malignant tumors and have shown that these
molecules can indicate poor prognosis (11-16). Moreover, only a few studies have focused on EGFR in osteosarcoma (36,37). Nonetheless, no previous study has simultaneously examined EGFR and its four downstream signaling molecules in osteosarcoma. Our results showed positive EGFR expression in 26 cases (55.3%), with cytoplasmic expression in 23 cases and membranous expression in 3 cases. Previous studies showed variable results with respect to the expression pattern of EGFR, ranging from 81% EGFR expression only in the cytoplasm of osteosarcoma to 57% of non-small cell lung cancer cases expressing membranous EGFR (38,44). In our study, cytoplasmic and membrane expression of EGFR showed a statistical correlation with Erk expression. This result supports previous studies that have suggested that Erk is a downstream signaling molecule of EGFR (1,2,5). Moreover, the possible role of EGFR should now be considered in osteosarcoma because real-time PCR showed an increased fold expression of EGFR in two of three osteosarcoma cell lines (HS3.7 and MG63). However, p-EGFR and Akt expression was not observed in any osteosarcoma case. In addition, placental tissue, which was used as a control tissue, also showed a negative result for Akt expression. We
used various staining protocols and repeated our immunohistochemical staining of p-EGFR and Akt; however, the staining results were not significantly different from one another. In real-time PCR, the two cell lines which showed increased EGFR expression also showed increased Akt expression. Although the number of cell lines in our study was small, a relationship between EGFR and Akt appears to exist.

In our study, Erk and survivin expression were statistically correlated with survival of patients and this finding is consistent with previous studies that have suggested that these molecules are prognostic factors that indicate a poor prognosis (26-28,31-34). In particular, survivin expression was correlated with Erk and Stat-3 expression and this supports the idea that...

| Table II. Clinicopathological data and expression of EGFR, Stat-3, Erk and survivin. |
|--------------------------------------------------|--|--|--|--|
| EGFR | Stat-3 | Erk | Survivin |
| Total | + (26) - (21) P-value | + (17) - (30) P-value | + (12) - (35) P-value | + (20) - (27) P-value |
| Age ≤25 | 32 | 17 | 15 | 0.758 | 10 | 22 | 0.344 | 10 | 22 | 0.288 | 15 | 17 | 0.529 |
| >25 | 15 | 9 | 6 | 7 | 8 | 2 | 13 | 0 | 0 | 0 | 5 | 10 |
| Gender Male | 25 | 14 | 11 | 1.000 | 8 | 17 | 0.558 | 9 | 16 | 0.103 | 11 | 14 | 0.831 |
| Female | 22 | 12 | 10 | 9 | 13 | 3 | 19 | 9 | 13 |
| Metastasis Present | 8 | 5 | 3 | 0.715 | 3 | 5 | 1.000 | 3 | 5 | 0.403 | 5 | 3 | 0.258 |
| Absent | 39 | 21 | 18 | 14 | 25 | 9 | 30 | 15 | 24 |

Pearson's Chi-square test. *Fisher's exact test.

Figure 3. Real-time PCR result of EGFR. Increased EGFR level is observed in two out of the three osteosarcoma cell lines, HS3.7 and MG63.

Figure 4. Mutation analysis of EGFR. The sequenced kinase domain from exons 18 to 21 in the cell lines show a point mutation which changed A to G at codon 54,031 at exon 20. However, it did not lead to a change in the amino acid (glutamine) at this position.
survivin is a downstream signaling molecule of Erk and Stat-3 (7,8,39). One study suggested that survivin is regulated via Erk through the PI3K signaling pathway. The authors of this study observed decreased survivin expression after using an Erk inhibitor (40). Furthermore, another study showed inhibition of survivin expression by using a drug that downregulates the Stat-3 signaling pathway in head and neck cancer (7). Therefore, Erk inhibiting or Stat-3 inhibiting drugs in osteosarcoma patients could potentially considerably increase patient survival rate. Our results showed Stat-3 expression in 17 cases of osteosarcoma, but we found no statistical correlation between EGFR expression and the clinicopathological data. One previous study has shown Stat-3 expression in bone and soft tissue tumors, but these studies did not find Stat-3 expression in the three osteosarcoma cases included (41).

The discrepancy between EGFR and Stat-3 expression may be as a result of the following. First, EGFR activation is not an essential step for Stat-3 signal transduction in osteosarcoma. In addition, some studies have described EGFR-independent activation of Stat-3 in vivo and in vitro (18,19). Second, other EGFR transduction pathways such as PI3K or the Ras/Raf/mitogen-activated protein kinase pathway possibly could be activated instead of the JAK/STAT signaling pathway. In fact, our results showed a statistical correlation between EGFR and Erk expression. However, the indirect effect of Stat-3 on patients via an increased expression of survivin should be considered.

We found one point mutation (A to G) at codon 54301 in exon 20 of the EGFR gene that did not lead to a change in the amino acid sequence. Previous studies of EGFR gene mutations in osteosarcoma found a point mutation at codon 863 in exon 21, E829E and R831C in exon 21 (36,41). Of all these mutations, only one mutation (R831C in exon 21) resulted in an amino acid change from arginine to cysteine. Accordingly, the type and location of mutations may have different spectrums in osteosarcoma. Previously, EGFR targeted therapies, as well as tyrosine kinase inhibitors, have been clinically used to treat many kinds of cancers (42,43). In addition, non-small cell lung carcinoma patients with EGFR mutations present in the cancer show a favorable clinical prognosis that is associated with an increased sensitivity to tyrosine kinase inhibitors (44-46). Considering the presence of EGFR mutations and the positive expression of EGFR, osteosarcomas may also be sensitive to tyrosine kinase inhibitors.

In conclusion, expression of Erk and survivin in osteosarcoma may indicate an unfavorable patient prognosis and an EGFR mutation may be a variable event in osteosarcoma. Moreover, trials of Erk- or Stat-3-inhibiting drugs should be considered in osteosarcoma patients with survivin expression. Nevertheless, a large-scale EGFR mutation evaluation in osteosarcomas is needed to determine their potential sensitivity to molecular targeting therapies.

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References


