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COPY NUMBER GAINS IN *EGFR* AND COPY NUMBER LOSSES IN *PTEN* ARE COMMON EVENTS IN OSTEOSARCOMA TUMORS

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Abstract

Background—Osteosarcoma cell lines and tumors have been shown to express *EGFR* and harbor amplifications at the *EGFR* locus. In this study, we further analyze the genomic features of *EGFR* in osteosarcoma tumors and investigate whether they correlate with *PTEN* expression and copy number status.

Methods—*EGFR* and *PTEN* expression were assessed by immunohistochemistry (n=28), and copy number alterations at the *EGFR* and *PTEN* loci were surveyed using Affymetrix® 50K single nucleotide polymorphism (SNP) arrays (n=31) and fluorescence in situ hybridization (FISH) (n=27). The *EGFR* tyrosine kinase domain was sequenced to survey for activating mutations (n=34). In addition, we assessed *EGFRvIII* expression using RT-PCR (n=24). Results were correlated with available clinical information on 59 patients, with a median age of 14.1 years (range, 5–23 years) and median follow up of 4.4 years.

Results—*EGFR* expression was detected in the majority of osteosarcoma tumors surveyed (23/28; 82%). SNP arrays revealed evidence of frequent copy number gains at 7p11.2 and losses at 10q23.21. A sizeable subset (16/27 cases; 59%) showed gains at the *EGFR* locus using FISH – amplification in 4/27 (15%) and balanced chromosome 7 polysomy in 12/27 (44%), and 12 cases showed deletions at the *PTEN* locus – biallelic deletions in 4/27 (15%) and monoallelic deletion in 9/27 (33%). No activating mutations in the *EGFR* tyrosine kinase domain, *EGFRvIII* expression, or association with clinical findings were detected.

Conclusion—*EGFR* expression and genomic gains at the *EGFR* locus are prevalent in osteosarcoma tumors, which also commonly harbor deletions at the *PTEN* locus.

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Keywords

childhood cancer; osteosarcoma; epidermal growth factor receptor; tyrosine kinase; EGFRvIII; PTEN; single nucleotide polymorphism array

INTRODUCTION

The epidermal growth factor receptor (EGFR; ErbB1) is a cell membrane receptor with intrinsic protein tyrosine kinase activity that has been the subject of rigorous investigation in view of its involvement in several human cancers and its potential as a target of therapy.¹ Several classes of anti-EGFR agents are currently approved for clinical use or in advanced clinical trials¹ in adult patients with cancer. Phase I clinical trials of EGFR tyrosine kinase inhibitors, such as gefitinib, have included children with refractory solid tumors, including patients with osteosarcoma.^{2, 3} Similar phase I trials of gefitinib in adult patients did not include osteosarcoma patients.^{4, 5} Activation of EGFR in normal cells is induced by ligand binding, which leads to tightly regulated stimulation of proliferative and survival cell signaling pathways, most notably the phosphatidylinositol 3-kinase (PI3K)–AKT pathway.^{6, 7}

Various types of EGFR alterations have been detected in human cancers, with varying clinical implications. Such alterations include aberrant EGFR expression, with or without gene amplification^{8–12}, expression of a mutant form with in-frame deletion of exons 2 to 7 (EGFRvIII)¹³, and point mutations in the tyrosine kinase domain. The EGFRvIII mutant protein lacks the ligand-binding domain but is capable of constitutive phosphorylation of tyrosine residues and subsequent activation of downstream signaling cascades¹⁴, and its expression appears to confer sensitivity to tyrosine kinase inhibitors.¹⁵ Response to EGFR inhibitors appears to correlate with expression of EGFRvIII and concurrent loss of *PTEN* in some neoplasms.^{16, 17} In addition, somatic mutations in the tyrosine kinase domain of EGFR have been found to correlate with response to tyrosine kinase inhibitors in a subset of patients with non-small cell lung carcinoma.^{18–20} Furthermore, increased EGFR gene copy number has been found to be associated with higher response rates to gefitinib and lower progression rates in patients with lung²¹ and colon²² carcinoma.

Osteosarcoma is the most common primary malignant bone tumor in children and adolescents.^{23, 24} Advances in osteosarcoma therapy over the past several decades have enhanced patient outcomes, with most effective regimens currently including neoadjuvant and adjuvant chemotherapy coupled with local control that usually consists of limb-sparing surgery.²⁵ However, outcome remains poor for most patients with metastatic or recurrent osteosarcoma and even for some patients with localized disease at presentation, despite intensification of initial chemotherapy and/or the use of second-line chemotherapeutic agents.^{26–32}

Among childhood malignancies, expression of EGFR has been reported in Wilms tumor³³, and it was recently found to correlate with the embryonal subtype of rhabdomyosarcoma³⁴. In osteosarcoma, *in vitro* data from early passage osteosarcoma cells demonstrate constitutive EGFR phosphorylation whose abrogation leads to growth inhibition and apoptosis.^{35, 36} Furthermore, recent studies have demonstrated EGFR expression in osteosarcoma tumors.^{37, 38} Interestingly, EGFR expression appears to be associated with favorable clinical outcome in patients with osteosarcoma.³⁷

In this study, we assess *EGFR* expression, copy number status, and tyrosine kinase domain mutations in a large group of osteosarcoma tumors. Furthermore, because loss of *PTEN* has been identified as an important molecular determinant of response to EGFR tyrosine kinase

inhibitors in some cancers^{17, 39}, we investigate whether these findings correlate with *PTEN* expression and copy number status, as well as with clinical features and disease outcome.

MATERIALS AND METHODS

Tumor samples and patient study group

This study was approved by the St Jude Children's Research Hospital (SJCRH) Institutional Review Board. A total of 80 osteosarcoma cases were utilized, as summarized in Table S1. Due to limitations in available tumor material, only subsets of these 80 osteosarcoma tumor samples were amenable for use in the different experiments performed in this study. All tumors fulfilled the diagnostic criteria for osteosarcoma as defined in the World Health Organization classification.²³ Formalin-fixed, paraffin-embedded (FFPE) tumors (n=28) were incorporated into tissue microarrays (TMA) using a manual arrayer (Beecher Instruments, Silver Spring, Maryland, USA). A minimum of three cores representing viable, non-decalcified osteosarcoma tumor tissue were collected from donor blocks. Cores from paraffin-embedded normal tissues were distributed in each of the TMA blocks as controls. Adequacy of tumor viability (<20% necrosis) and histologic subtype were assessed on all frozen tumor samples by routine microscopic examination of a representative section.

Clinical information was available on 59 patients. Only limited demographic and clinical data were available on the 21 remaining cases, which included patients who were referred for clinical and/or pathological consultation but were not admitted to or managed at SJCRH. Notably, the patients whose tumor material was used in this study were managed disparately on a variety of therapeutic protocols and nonprotocol regimens. The demographic and clinical features of the patient group are summarized in Table 1.

Immunohistochemistry for assessment of EGFR and PTEN expression

Expression of EGFR was assessed using a monoclonal anti-EGFR antibody (31G7, 1:10; Zymed, San Francisco, California, USA) on the automated Ventana Benchmark XT immunostainer (Ventana Medical Systems, Tucson, Arizona, USA) using the manufacturer's deparaffinization, antigen retrieval, and detection reagents as described previously.⁴⁰ Expression of PTEN was assessed using a monoclonal anti-PTEN antibody (6H2.1, 1:400; Dako, Carpinteria, California, USA)⁴¹ on a semi-automated Dako Automated Stainer (Dako). For PTEN immunostaining, antigen retrieval was carried out manually by placing tissue sections in plastic Coplin jars containing preheated TRIS/EDTA pH 9 solution in a household vegetable steamer (Sunbeam-Oster, Model Sunbeam 4713/5710, 900 W) for 30 minutes. Subsequent steps were performed using the Dako Automated Stainer (Dako) at room temperature. We used 3,3'-diaminobenzidine/H₂O₂ (Dako) or 3-amino-9-ethyl carbazol as chromogens and hematoxylin as the counterstain. All immunostains were performed in duplicate. Negative control immunostains were performed as indicated above, less the primary antibody. Tonsil tissue (full sections and scattered cores in TMA blocks) served as positive control for both the EGFR and PTEN immunostains, with the former staining the squamous epithelium and the latter staining a subset of lymphocytes.

Immunostaining results were assessed as described previously.³⁴ Immunostaining intensity was estimated for each tissue core as follows: 1, weak; 2, moderate (equivalent to immunostaining by positive control in TMA); or 3, strong. The percentage of tumor cells positive for EGFR and PTEN was estimated by counting at least 500 tumor cells in each core. Results were scored by multiplying the percentage of positive cells by the estimated intensity of immunostaining (score=percentage × intensity; range, 0 to 300). The core with the highest score determined the final value for each case. For the purposes of statistical analysis, a score of >20 was used to define positivity based on a data distribution plot (not

shown), and tumors were considered to have strong EGFR or PTEN expression when the immunostaining score was ≥ 120 .

Single nucleotide polymorphism (SNP) array analysis for assessment of EGFR and PTEN copy number status

SNP analysis was performed using the Affymetrix Mapping 50K Xba-I 240 array (Affymetrix, Santa Clara, California), which interrogates 59,015 SNPs across the whole human genome.⁴² DNA quality was confirmed by UV spectrophotometry and agarose gel electrophoresis. DNA samples were processed by the core laboratory in the Hartwell Center for Bioinformatics and Biotechnology at SJCRH using the recommended Affymetrix protocol. Briefly, 250 ng of genomic DNA from 31 frozen osteosarcoma tumors from 30 patients was digested to completion with Xba-I restriction endonuclease then ligated to an adapter containing a generic primer sequence. Three aliquots (25 ng each) of ligated DNA were amplified using conditions to enrich for 250 to 2000 bp DNA fragments and then pooled and purified. DNA was fragmented using DNase I and end-labeled with biotinylated nucleotide using terminal deoxynucleotidyl transferase. The labeled targets were added to a hybridization cocktail containing probe array controls and blocking agents (human Cot-1 and herring sperm DNA) and incubated overnight at 48°C on a GeneChip array. After hybridization, arrays were stringently washed and stained with Streptavidin Phycoerythrin (SAPE, Invitrogen - Molecular Probes, Carlsbad, California, USA) using the Affymetrix Fluidics Station 400. Arrays were scanned using the Affymetrix GeneChip Scanner 3000 and genotype calls determined using the Affymetrix Gene DNA Analysis Software (GDAS) version 3.0.

Fluorescence in situ hybridization for confirmation of EGFR and PTEN copy number status

Dual-color FISH was performed on 4 μ m paraffin-embedded TMA sections. Probes were derived from BAC clones (Invitrogen; CHORI, Oakland, California, USA) and labeled with either FITC or Rhodamine fluorochromes. Target probes were paired with control probes from the opposite chromosomal arm. For *EGFR* copy number status assessment, a FITC-conjugated probe that is specific for the *EGFR* locus on chromosome 7p11.2 (RPC11-148P17; Invitrogen) was paired with a rhodamine-conjugated control probe specific for the *CFTR* locus on chromosome 7q31.2 (RP11-460J21 & CTB-133K23; Invitrogen). For *PTEN* copy number status assessment, a rhodamine-conjugated probe that is specific for the *PTEN* locus on chromosome 10q23.21 (RP11-380G5; Invitrogen) was paired with a FITC-conjugated control probe specific for 10p11.21 (RP11-254A5 & RP11-32212; Invitrogen). Nuclei were counterstained with DAPI (1000ng/ml) (Insitus) for viewing on a Nikon Eclipse E800 fluorescence microscope. Images were captured and processed with an exposure time ranging from 0.5–2 seconds for each fluorochrome using Cytovision v3.6 software (Applied Imaging, San Jose, California, USA).

Cores in which >90% of nuclei showed hybridization signals were considered informative, and those were scored for the number of fluorescent signals by counting 100 non-overlapping intact nuclei. FISH patterns were defined as follows: balanced disomy refers to the identification of dual signals from both the test and control probes; balanced polysomy refers to similar gains in copy numbers for both the test and control probes; gene amplification refers to unbalanced gains in copy number for the test probe.

Mutation analysis of the EGFR tyrosine kinase domain

Genomic DNA was extracted from 34 frozen osteosarcoma tumors and used to amplify EGFR exons 18, 19, 20, and 21 using primer sequences and PCR conditions that were kindly provided by Dr. Matthew Meyerson (Dana-Farber Cancer Institute, Boston, Massachusetts, USA) and available upon request. Following amplification, all PCR

products were purified using the Qiaex II gel extraction kit (Qiagen, Valencia, CA, USA) or Microcon-100 purification columns (Millipore, Billerica, MA, USA). Purified DNA was then sequenced in both the sense and antisense directions on all cases by Big Dye Terminator (v.3) Chemistry using PCR primers on Applied Biosystem 3700 DNA Analyzers.

Assessment of EGFRvIII expression

RNA of adequate quality was extracted from 24 frozen osteosarcoma tumors and used to detect EGFRvIII mutant expression as described previously by Ji et al¹⁵. Briefly, RNA was retrotranscribed into first-strand cDNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, California, USA). PCR was then used to amplify the region spanning *EGFR* exons 1 and 8. The RT-PCR products – 128 bp (EGFRvIII) and/or 929 bp (WT EGFR) – were visualized using 2% agarose gel electrophoresis.

Statistical analysis

Exact Pearson chi-square tests were used to test for associations among categorical variables. The Kaplan-Meier method was used to estimate event-free survival (EFS), defined as the time interval from diagnosis to first event (relapse, progression, second malignancy, or death from any cause) or to last follow up for patients without events.

Copy number and LOH analyses of the 50K SNP array data were performed using dChipSNP as described previously.^{43, 44} For copy number analysis, data were first normalized to a baseline array having median signal intensity at the probe intensity level with the invariant set normalization method. After normalization, the signal values for each SNP in each array were obtained with a model-based (perfect-match/mismatch difference model) method. Signal intensities for each SNP in each tumor were then compared with those from a reference set of 61 normal samples and copy numbers inferred using a hidden Markov Model (HMM).^{45, 46} LOH regions were identified using a HMM-based method and the 61 unpaired normal samples.

RESULTS

EGFR expression is common in osteosarcoma tumors

Immunostaining results are illustrated in Figure 1. EGFR expression was identified using immunohistochemistry in 23 of 28 (82%) osteosarcoma tumors, of which 19 of 23 (83%) exhibited strong expression. The average EGFR immunostaining score was 149 (median: 135; range among EGFR-positive cases: 50–300). The immunostaining pattern was membranous or membranous and cytoplasmic in all tumors.

PTEN immunostaining was identified in 19 of 28 (68%) osteosarcoma tumors. The average PTEN immunostaining score was 122 (median: 110; range among PTEN-positive cases: 30–300). Of the 23 EGFR-positive osteosarcoma tumors, 9 (39%) were PTEN-negative; however, there was no correlation between EGFR expression PTEN expression ($p=0.64$). Interestingly, two of the 28 osteosarcoma tumors exhibited chondroblastic morphology, and both were EGFR-positive and PTEN-negative.

Inferred genomic gains at 7p11.2 and losses at 10q23.21 are frequent in osteosarcoma tumors

Using SNP arrays, we inferred the presence of frequent copy number gains in the region of the *EGFR* locus and frequent losses in the region of the *PTEN* locus in osteosarcoma tumors, as compared to the reference set (Figure 2). Of 31 tumors analyzed, at least 16 osteosarcoma tumors exhibited evidence of copy number gains in the chromosome 7p11.2

region whereas at least 12 exhibited evidence of copy number losses in the 10q23.21 region. Notably, 13 tumors (42%) harbored concurrent evidence of copy number gains at 7p11.2 and copy number losses at 10q23.21. In addition, 11 of 31 (35%) of osteosarcoma tumors analyzed show LOH at the *PTEN* locus, whereas only 2 cases show LOH at the *EGFR* locus.

***EGFR* amplification is detected using FISH in a subset of osteosarcoma tumors**

To begin understanding the molecular mechanisms underlying *EGFR* expression in osteosarcoma tumors and validate the data obtained from SNP arrays, we performed FISH on 28 osteosarcoma tumors to estimate the copy number status of the *EGFR* locus on chromosome 7p11.2. Of 27 (27/28, 96%) osteosarcoma tumors with interpretable hybridization signals, 4 (15%) showed evidence of *EGFR* gene amplification (Figure 3A). In addition, gain of material at 7p11.2 resulting from balanced chromosome 7 polysomy was identified in 12 (44%) tumors, monoallelic deletion was identified in 1 (4%) tumor, and 10 (37%) tumors had balanced disomy.

All osteosarcoma tumors with *EGFR* amplification had high-level *EGFR* expression. However, of the 12 tumors with evidence of balanced polysomy, 9 had *EGFR* expression (6 strong, 3 weak) and 3 lacked *EGFR* expression. There was no correlation between *EGFR* copy number status, as assessed by FISH, and protein expression, as assessed by immunohistochemistry.

We also performed FISH on 28 osteosarcoma tumors to estimate the copy number status of the *PTEN* gene at 10q23.21. Of 27 (27/28, 96%) osteosarcoma tumors with interpretable hybridization signals, 4 (15%) tumors had biallelic deletion at the *PTEN* locus, 9 (33%) tumors had monoallelic deletion at the *PTEN* locus, 3 (11%) tumors showed balanced polysomy, 5 (19%) had a mixed pattern whereby subsets of tumor cells harbored monoallelic deletion of *PTEN* whereas others harbored balanced polysomy, and 6 (22%) had balanced disomy (Figure 3B). Because it was uncertain whether cases that demonstrated a mixed pattern should be assigned to the “loss” or “gain” categories, these cases were not included in subsequent statistical analyses.

When we examined the relation between *PTEN* copy number status and expression, we found a significant association between biallelic deletions of *PTEN* and negative or weak *PTEN* expression ($p=0.041$). In addition, osteosarcoma tumors with either monoallelic or biallelic deletion of *PTEN* tended to have weak or negative *PTEN* expression ($p=0.057$). Of note, we identified a correlation between *EGFR* amplification and monoallelic deletion of *PTEN* ($p=0.05$) in our study group. Interestingly, however, there was no correlation between *EGFR* amplification and biallelic deletion of *PTEN*.

***EGFRvIII* is not expressed in osteosarcoma tumors**

EGFRvIII, a mutant form of *EGFR* with in-frame deletion of exons 2–7 (801 bp), constitutively activates the PI3K signaling pathway and confers sensitivity to *EGFR* tyrosine kinase inhibitors¹⁶. Using RT-PCR and primers specific for *EGFR* exons 1 and 8, we found no evidence of deletion of exons 2–7 in any of the 24 osteosarcoma tumors surveyed. Gel electrophoresis showed variable intensity ~929 bp amplification bands, as expected for the wild type *EGFR* transcript (Figure 4).

Mutation analysis of the *EGFR* tyrosine kinase domain reveals no evidence of activating mutations

Certain mutations in the *EGFR* tyrosine kinase domain lead to constitutive activation and confer susceptibility to certain tyrosine kinase inhibitors.¹ To determine whether such

mutations are present in osteosarcoma tumors, we analyzed exons 18–21, which encode for the EGFR tyrosine kinase domain. Interestingly, known *EGFR* polymorphisms without resulting amino acid changes were noted in most cases, as summarized in Table S2. However, we did not identify known mutations that have been associated with constitutive EGFR activation in the 34 osteosarcoma tumors analyzed.

Correlation of *EGFR* and *PTEN* expression and copy number status with clinical features

We found no correlation between EGFR or PTEN expression and copy number status with presenting clinical features and outcome data, which included disease stage at presentation, primary tumor's histologic response to chemotherapy (Rosen grade I and II vs III and IV), and survival.

DISCUSSION

In cancer cells, EGFR aberrations impact a variety of cell signaling pathways, notably the PI3K-AKT and JAK/STAT pathways.¹ The PI3K-AKT pathway plays an important role in modulating the mammalian target of rapamycin (mTOR) and is effectively inhibited by PTEN.⁴⁷ *In vitro* data indicate that EGFR and AKT signaling play a role in the pathogenesis of osteosarcoma^{35, 36, 48}. Our data additionally demonstrate that in osteosarcoma tumors there is an overall genomic gain in *EGFR* and loss in *PTEN*. Such an imbalance implies that growth-promoting pathways influenced by EGFR, notably PI3K-AKT/mTOR, might be constitutively upregulated in osteosarcoma. In fact, AKT activation or functional loss of PTEN, the major negative regulator of PI3K, has been found to comprise an important cause of resistance to the anti-EGFR agent, gefitinib.⁴⁹ Although further studies are needed to explore the downstream influence of EGFR aberrations in osteosarcoma tumors, evidence in support of such a contention includes data showing that inhibition of mTOR⁵⁰ and PI3K-AKT⁵¹ have adverse effects on a murine model of osteosarcoma and osteosarcoma cell lines, respectively. Interestingly, in a survey of 32 osteosarcoma tumors, we did not identify evidence of STAT3 activation (J. Khoury, unpublished data), arguing against a prominent role for the JAK/STAT pathway. Taken together, these data appear to indicate that osteosarcoma tumors could be biologically dependent on EGFR-modulated cell signaling pathways, especially PI3K-AKT/mTOR, a fact that might be exploitable for therapeutic purposes.

In addition to demonstrating that most osteosarcoma tumors harbor genomic gains at the *EGFR* locus using SNP arrays and FISH, our data also show that loss of *PTEN* is a common occurrence in osteosarcoma tumors. We were intrigued to identify a correlation between *EGFR* amplification and monoallelic loss of *PTEN*. Indeed, there is mounting evidence indicating that loss of *PTEN* in tumors with EGFR expression is associated with resistance to anti-EGFR tyrosine kinase inhibitors.³⁹ *In vitro* evidence suggests that such resistance can be overcome by coupling anti-EGFR agents with mTOR inhibitors.⁵² Novel therapeutic approaches of this kind appear warranted if anti-EGFR therapy is to be investigated in osteosarcoma patients. Furthermore, since it appears that in a sizeable subset (44%) of osteosarcoma tumors the mechanism of copy number gain at the EGFR locus involves balanced chromosome 7 polysomy or gains in large segments of chromosome 7, further studies will be necessary to determine whether response to anti-EGFR therapy in osteosarcoma may be influenced by the mechanism of copy number gain.

Our data are in line with two recent studies that also demonstrate EGFR expression in a sizeable subset of osteosarcoma tumors, of which a subset also harbors gains at the EGFR locus.^{37, 38} Interestingly, one of those studies identified a correlation between EGFR expression and favorable clinical outcome.³⁷ In our study group, we did not identify a correlation between expression and copy number status of EGFR, or PTEN, and clinical

features. We speculate that the most likely causes include the non-uniformity of therapeutic regimens used to treat patients in our group, a limitation of this retrospective study, and the disparity in the number of pre-therapy versus post-therapy samples employed in the two studies.

In conclusion, data from this study using osteosarcoma tumor samples support *in vitro* and recent clinical data suggesting a role for the EGFR/PI3K-AKT pathway in osteosarcoma. Clinical investigative trials using approved and/or novel therapeutic agents that specifically target this pathway appear warranted.

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Figure 1. Panel illustrating EGFR and PTEN expression in osteosarcoma using immunohistochemistry. For EGFR, assigned scores for these illustrated cases were 300, 100, and 0. For PTEN, assigned scores for these illustrated cases were 300, 100, and 0.

Figure 2. Results of 50K SNP array analysis in 31 osteosarcoma tumors demonstrate frequent genomic gains at 7p11.2, which includes the *EGFR* locus, and frequent genomic losses at 10q23.21, which includes the *PTEN* locus, using log₂ ratio of tumor signal/mean signal of all 61 references for each SNP in each tumor case. In addition, loss of heterozygosity appears more common at 10q23.21 than at 7p11.2 (yellow: retention; dark blue: loss of heterozygosity; light blue: non-informative). These findings are matched with corresponding data (top panel) obtained using fluorescence in situ hybridization and immunohistochemistry where available. [A: amplification; CN: copy number; FISH: fluorescence in situ hybridization; IHC: immunohistochemistry; M: mixed pattern of monoallelic loss and polysomy; n: suboptimal hybridization; N: negative; p: balanced polysomy; P: positive; L1: monoallelic loss; L2: biallelic loss; LOH: loss of heterozygosity]

Figure 3.

(A) Fluorescence in situ hybridization demonstrating osteosarcoma tumor with EGFR gene amplification (green signal: EGFR test probe at 7p11.2; red signal: control probe at 7q31.2).

(B) Another osteosarcoma tumor harboring biallelic loss of PTEN (red signal: PTEN test probe at 10q23.21; green signal: control probe at 10p11.21); note the presence of chromosome 10 polysomy.

Figure 4.

Agarose gel electrophoresis of RT-PCR products using EGFR exon 1 and exon 8 primers. The wild type EGFR mRNA is expected to yield a 929bp amplicon whereas the EGFRvIII mutant mRNA, in which exons 2 to 7 are deleted, is expected to yield a 128bp amplicon. None of the cases analyzed in our group showed expression of EGFRvIII.

Table 1

Summary of demographic and clinical features

	Total (n=59)
Age (years)	
Median (range)	14.1 (5.01–23.5)
14	28
>14	31
Gender	
Female	26
Male	33
Race	
Non-White	22
White	37
Survival status	
Alive	41
Expired	18
Site of primary tumor	
Extremity	55
Axial	4
Disease at presentation (n=58)	
Local, resectable disease	48
Local, unresectable disease	1
Metastatic disease	10
Rosen grade [*] (n=53)	
1 or 2	22
3 or 4	31
Follow up (years)	
Median (range)	4.4 (0.5–14)

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	Total (n=59)
Outcome (%)	
5-Year overall survival	63.6 ± 10.3
5-Year event-free survival	57.0 ± 10.0

* As described previously^{53–55} - Grade I: <50% tumor necrosis; Grade II: 50%–90% tumor necrosis; Grade III: >90% tumor necrosis; Grade IV: 100% tumor necrosis.