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Ectopic Production of B-hCG and Loss of P16 as a Predictor of Outcome in Patients with Newly Diagnosed Osteosarcoma

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Abstract

Osteosarcoma is the most common malignant bone tumor in children and young adults and is associated with high mortality. We investigated the expression of β- hCG and P16 in osteosarcoma and correlated with outcomes.

Methods: Immunohistochemistry (IHC) for β-hCG was performed on diagnostic osteosarcoma specimens and post-treatment specimens. IHC for P16 was performed on diagnostic specimens.

Results: Median age was 32. Median progression free survival (PFS) was 11.5 months. Median overall survival (OS) was 38.0 months. Positive β-hCG staining on diagnostic specimens did not correlate with percent tumor necrosis, 2 year PFS or OS. Patients with a post-treatment β-hCG staining of ≥ 50% had a median PFS of 6.1 months vs 19.2 months in patients with β-hCG less than 50% (p = 0.03). Patients with a post-treatment β-hCG staining ≥ 50% also demonstrated a trend toward shorter median OS (17.1 months vs 19.2). There was no statistically significant relationship between P16 staining on diagnostic osteosarcoma specimens and post- treatment percent tumor necrosis. Patients with negative P16 staining on diagnostic specimens had a lower 2 year PFS compared to positive P16 staining (2 year PFS 0% vs 71%), p=0.022. There was a trend toward worse 2 year OS in patients with P16 negative diagnostic specimens compared to patients with P16 positive tumors, 22% vs 86%.

Discussion: We have demonstrated feasibility and utility in examining P16 and β-hCG in osteosarcoma. We found that post-treatment β-hCG expression correlated with poorer outcomes, specifically worsened PFS. In congruence with previous reports, negative P16 staining confers worse outcomes.

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Introduction

Osteosarcoma while considered a rare cancer, is the most common malignant bone tumor in children and young adults [1] and comprises 28% of the bone cancers diagnosed in adults over the age of 40 [2]. Osteosarcoma is associated with a relatively high mortality rate and a 5 year overall survival of only 66.7% [1]. Several factors have been demonstrated to be prognostic including tumor location, size, patient age, metastatic disease, histological response to chemotherapy and surgical outcomes [3-5]. However there is little data on individualized tumor characteristics for predicting or monitoring response to chemotherapy. Ectopic production of β- hCG (human chorionic gonadotropin), by osteosarcomas is an uncommon phenomenon that has rarely been documented and few case reports have noted a trend towards poor outcomes [6-8]. Inactivation of P16 has also been associated with continuous cell proliferation in numerous malignancies and may correlate with worse outcomes in osteosarcoma [9,10]. We recently observed a case of a recurrent osteosarcoma associated with high levels of serum β-hCG, which normalized after tumor resection. Immunohistochemical staining for β-hCG established the tumor to be the source of the elevated serum maker. We have further investigated the expression of β-hCG and P16 in osteosarcoma tumors diagnosed at our institution and correlated these findings with clinical outcomes.

Materials and Methods

Thirteen adult patients with available pathologic specimens were diagnosed with osteosarcoma at our institution between 2006 and 2014. Retrospective chart review was conducted on included

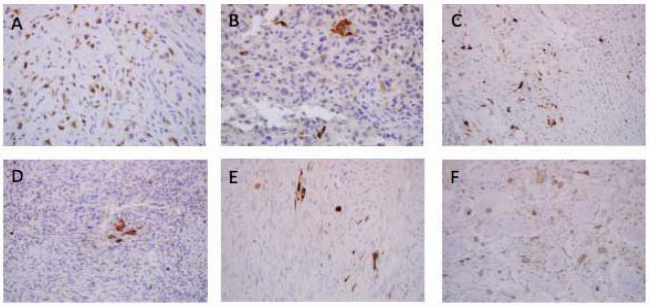

Figure 1: Representative images of immunohistochemistry and designated categories of the analyzed biomarkers. A, P16 positive staining. B, Positive β-hCG staining (<10% category) of diagnostic specimen. C, Positive β-hCG staining (≥10- 50% category) of diagnostic specimen. D, Positive β-hCG staining (<10% category) of post-treatment specimen. E, Positive β-hCG staining (≥10- 50% category) of post-treatment specimen. F, Positive β-hCG staining (≥50% category) of post-treatment specimen.

patients and demographic, clinical, pathological, radiological and laboratory data was collected. Previously stored formalin fixed and paraffin embedded blocks from available pre- treatment diagnostic specimens and the designated study pathologist reviewed post-treatment surgical specimens of included patients, and the most representative block was chosen for sectioning. The slides with the greatest proportion of tumor were then selected for staining. Two representative slides from the diagnostic specimen (one for β-hCG and one for P16 staining) and one representative slide from the post treatment resection specimen (only for β-hCG) were selected per subject. Immunohistochemistry staining for β-hCG was performed on the formalin fixed, paraffin embedded tissue sections of the diagnostic osteosarcoma specimens and post treatment surgical resection specimens. Rabbit polyclonal anti-human β-hCG antibody (Dako, Carpinteria, California) was utilized with pH adjusted (pH 6.0) antigen retrieval. Placenta was used as positive control. Stained slides were examined by the study pathologist and analyzed for cytoplasmic staining intensity (absent or present) and frequency of expression (categorized as 0%, <10%, 10-50% or ≥ 50%) was reported. Both intensity and frequency of β-hCG expression was analyzed, as there is no established grading system for β-hCG staining pattern in osteosarcoma (Figure 1). Immunohistochemistry staining for P16 was performed on the formalin fixed, paraffin embedded tissue sections of the diagnostic osteosarcoma specimens. Mouse monoclonal anti-human P16INK4a antibody (Biocare Medical, LLC, Concord, California) was utilized with pH adjusted (pH 9.0) antigen retrieval. The positive control was cervical cancer specimens. Tonsil tissue microarrays were used for negative control. Both intensity and frequency of P16 expression was analyzed and reported. Specimens were classified as negative (absent) or positive (present) for P16 nuclear expression (Figure 1). Positive staining for P16 included specimens with ≥ 30% nuclear staining. This threshold was chosen based on prior reports of P16 staining in osteosarcoma [10,11]. Assessment of tumor necrosis is reported as a percentage and was obtained from post-treatment surgical specimen pathology report on chart review. Progression-free and overall survival was analyzed using Kaplan-Meier curves. Subgroups were compared using the log rank test. Comparisons of biomarkers between p16 positive and negative subgroups were done using the Wilcoxon rank sum test.

Results

Thirteen patients with available pathology were diagnosed with osteosarcoma at our institution between 2006 and 2014. The median

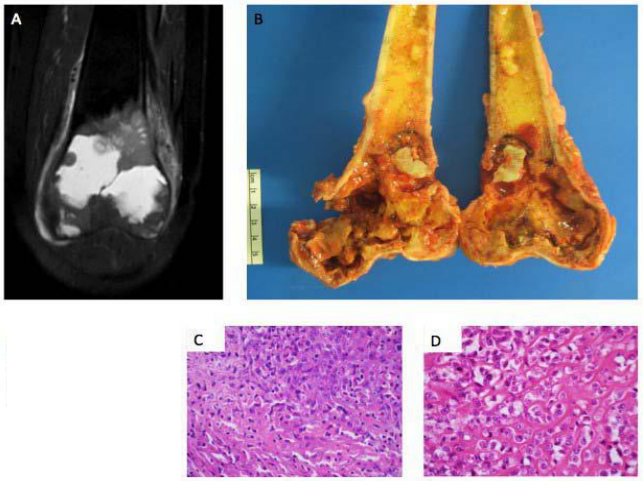

Figure 2: Index patient: A, MRI left femur. B, gross specimen. C, Diagnostic open biopsy hematoxylin-eosin. D, Post-treatment hemoatoxylin-eosin.

Table 1: Text Here.

Patient #	Age	Sex	Location	Subtype
101	51	F	Femur	Secondary (fibrous dysplasia)
102	42	F	Knee	Parosteal
103	28	F	Femur	Conventional (Fibroblastic)
104	28	F	Knee	Conventional
105	34	M	Shoulder	Conventional (Osteoblastic)
106	22	F	Knee	Conventional
107	60	M	Femur	Dedifferentiated parosteal
108*	30	F	Femur	Conventional (epithelioid features)
109	20	M	Chest wall	Conventional (Chondroblastic)
110	23	M	Femur	Telangiectatic
111	64	F	Palate	Conventional (Chondroblastic)
112	32	M	Femur	Conventional (Fibroblastic)
113	36	M	Skull	Secondary (radiation)

age at diagnosis was 32 (range 20-64) including 7 females and 6 males. The median follow up time was 26.7 months (range 5.2 – 91.0). Median progression free survival (PFS) was 11.5 months. Median overall survival (OS) was 38.0 months. Further characteristics of tumors described in (Table 1).

β-hCG

Ten patients had diagnostic specimens available for β-hCG staining. Of these 10 patients included, 7 had post-treatment resection specimens available with percent tumor necrosis detailed on final pathology report. There was no statistically significant relationship between β-hCG staining on the diagnostic osteosarcoma specimen and percent tumor necrosis on resection specimen. Positive β-hCG staining on the diagnostic specimen (n=6) did not correlate with worse 2 year PFS (p=0.45) or OS (p=0.90). Eight patients had a post-treatment tissue specimen available for β-hCG staining. Patients with negative staining for β-hCG (n=4) had a 2 year PFS of 50% compared with patients with positive β-hCG staining (n=4) whom had a 2 year PFS of only 25%. There was a trend toward worse 2 year PFS and OS in patients with positive β- hCG staining on post-treatment tissue, however the difference was not statistically significant. Given there is no established grading system for β-hCG staining pattern in

osteosarcoma, we also analyzed 2 year PFS and OS in patients with post- treatment β-hCG staining of ≥ 50% (n=2) compared to those with negative or < 50% staining. Patients with post-treatment β-hCG staining ≥ 50% had a median PFS of 6.1 months vs 19.2 months in patients with β-hCG staining less than 50% (p = 0.03). Patients with post-treatment β-hCG staining ≥ 50% also demonstrated a trend towards shorter median OS of 17.1 months vs 19.2 months (Figure 3).

P16

Ten patients had diagnostic specimens available for P16 staining. Of these 10 patients, 7 had post-treatment resection specimens available with percent tumor necrosis detailed on final pathology report. Five out of 7 patients had positive staining for P16 on pre-treatment diagnostic specimen. There was no statistically significant relationship between P16 staining on pre-treatment diagnostic osteosarcoma specimen and post-treatment percent tumor necrosis on resection specimen. Patients with negative P16 staining on the diagnostic specimen (n=3) did have a statistically significant lower 2 year PFS (0%, mean 10.9months) compared to those with positive (n=7) P16 staining (2 year PFS 71%, mean 25.9 months), p=0.022. There was a trend toward worse 2 year overall survival in patients with P16 negative diagnostic specimens compared to patients with P16 positive tumors, 22% vs 86%, however this did not reach statistical significance (p=0.25) (Figure 4).

Discussion

Our index patient was a 30 year old woman who presented with a left distal femur mass and an elevated serum β-hCG. Biopsy of the distal femur mass revealed a high- grade sarcoma most consistent with osteosarcoma with epithelioid features. Scattered tumor cells within the infiltrate did stain positive for β-hCG. Staging scans included CT chest which demonstrated two 2mm left lung nodules that were too small to characterize and a whole body bone scan which demonstrated increased activity in the distal left femur but no other focal abnormal activity elsewhere to suggest metastatic disease. Final staging of her tumor was stage IIa (T1N0M0G3). She initiated neoadjuvant chemotherapy with doxorubicin, cisplatin and high-dose methotrexate. Her β-hCG on C1D1 of therapy was 2288 mIU/mL and was trended throughout chemotherapy with an initial decrease to 1393 mIU/mL at week four and final pre-surgical β-hCG of 2417 mIU/mL at week 10. Restaging imaging prior to surgical resection demonstrated stable 2 mm left upper lobe lung nodules and no evidence of metastatic disease. MRI of the left femur however demonstrated extension of the osteosarcoma of the distal femur into the knee joint synovial soft tissues and an associated pathologic fracture that had progressed since initial imaging. She proceeded to surgical resection and underwent a left above knee amputation. Surgical pathology from resection demonstrated a high-grade osteosarcoma measuring 12 x 6 x 6cm with extension though the lateral meta- epiphyseal cortex into surrounding tissue and resulting in skin ulceration. Tumor showed approximately 40% chemotherapeutic response. Margins were negative. One lymph node was removed with no evidence of sarcoma. Final pathology staging was pT2pN0Mx (Figure 2). Three weeks after amputation, she presented to the emergency room with cough and fever. β-hCG remained detectable in serum at 26.1 mIU/mL. CT chest demonstrated a large multiloculated pleural effusion and low density lung mass occupying the entire left hemithorax with mass effect concerning for empyema, rapidly growing lung and pleural based masses as well as new metastatic nodules in the right lung. Left video assisted thoracoscopic surgery demonstrated a very small amount of free pleural fluid and a white firm necrotic appearing material was occupying much of the space. Pleural biopsy pathology was positive for high grade sarcoma.

Chorionic gonadotropin is a hormone produced by trophoblast that promotes growth of a developing embryo during pregnancy or less commonly, promotes growth and invasion in gestational trophoblastic disease[12]. Ectopic production of β-hCG by non-trophoblastic tumors has been reported [6], however our review of the literature found that the ectopic production of β-hCG by osteosarcomas has only been recognized in several case reports and small retrospective studies [8,13-18]. Masrouha et al. [7] reviewed histopathology slides of thirty-two patients with osteosarcoma and retrospectively stained the slides for β-hCG. Five of the thirty- two patients' specimens stained positive for β-hCG. In these five patients there was a trend toward poor outcomes (which they define as initial presence or development of metastatic disease or tumor recurrence) and clinically more aggressive tumors (as defined by post-therapy percent necrosis). While there is a trend towards worse outcomes in patients whose tumor expresses β-hCG, more robust data is needed to further characterize the significance of ectopic expression of β-hCG by the tumor and outcomes. Despite the ununiformed expression of β-hCG in osteosarcoma patients, it raises the question of the potential use of this marker in a subgroup of patients. Intact human chorionic gonadotrophin (hCG) is produced by normal placenta and germ cell tumors while epithelial tumors typically produce the free β subunit [19]. The intact heterodimeric hCG is part of normal pregnancy, developmental signaling and tissue differentiation; however Iles et al suggested that the free β subunit may have very different effects on the secreting tumors, specifically anti-apoptotic and pro-angiogenic influences. These properties may be related to the structure of β- hCG which is a member of the cysteine knot growth factor/TGFb superfamily and shares features similar to VEGF and TGFβ [20]. Butler et al. [21] demonstrated an increase in a bladder carcinoma cell population treated with β-hCG correlating with a decrease in apoptotic bodies using MTT assay in a dose-dependent fashion further supporting an anti-apoptotic effect of β-hCG. Several investigators have demonstrated a correlation between VEGF, hCG and angiogenesis in the placenta and developing ovarian follicles [22] as well in tumor angiogenesis [23,24]. The direct role of β-hCG in tumor angiogenesis remains to be more clearly defined.

There has also been further investigation into understanding the role of β-hCG in chemoresistance. Sahoo et al. [24] examined molecular pathways mediating continued tumor cell proliferation in hCG exposed cells despite chemotherapy. Tumor cells pre-exposed to hCG and then treated with chemotherapy (including 5-FU and etoposide) demonstrated increased viability and increased rates of proliferation compared to non-hCG exposed cells. They found the mechanism of chemoresistance does not appear to be driven by one mechanism alone but suggests a multifactorial explanation including evasion of apoptosis and maintenance of cytokines associated with tumorigenesis typically reduced with chemotherapy [25]. Our results demonstrate worse clinical outcomes in patients with post-treatment β- hCG tissue staining of ≥ 50% compared to those with negative or < 50% staining as defined by decreased 2 year PFS and OS. While we did not see a statistically significant influence of diagnostic β-hCG tumor expression on outcomes, there was a trend towards poorer outcomes in patients with any positive β-hCG on post-treatment specimens. These results do raise the question if increased expression of β-hCG by osteosarcoma tumors post-chemotherapy is

a marker or even mechanism of chemoresistance. Larger prospective investigation is warranted to confirm post-treatment β-hCG staining of osteosearcoma tumors as a prognostic tissue marker. We also note that abrogation of the G1 cell-cycle checkpoint occurs in a variety of malignancies and investigated the role of P16 in osteosarcoma on clinical outcomes.

Cyclin-dependent kinase 4 inhibitor referred to as P16INK4a (P16) is a major component of the G1 cell-cycle checkpoint, which also includes the retinoblastoma protein (pRB) and cyclin D1 [26]. Inactivation of P16 or pRB proteins by mutation, deletion, or promoter hypermethylation has been associated with continuous cell proliferation in numerous malignancies, and loss of P16 expression has been correlated with worse survival in osteosarcoma [9,10,27]. In a study conducted by Borys et al. [11] immunohistochemistry staining for P16 was performed on 40 specimens from patients with osteosarcoma. P16 expression correlated positively with post-therapy percent tumor necrosis. In a similar study conducted in a pediatric osteosarcoma population by Maitra et al, tissue from 38 patients were stained for P16 expression [10]. Sixteen percent demonstrated a loss of P16 expression and this absence significantly correlated with decreased overall survival. We have confirmed similar findings in our investigation with patients whose tumors had negative P16 staining at diagnosis having a statistically significant lower 2 year PFS and a trend towards shorter 2 year overall survival (22% vs 86%). We recognize there are several limitations to our study including that it was a retrospective analysis. P16 and β-hCG may be expressed heterogeneously within a tumor and not completely represented on the small biopsy specimen typically obtained to make the diagnosis of osteosarcoma lending to potential for false positives or negatives. In addition, our cohort size was smaller than anticipated due to limited availability of tissue specimens, which in many instances were obtained at outside hospitals prior to referral to our institution and then returned after pathology review.

Conclusion

In conclusion, we found that almost half of osteosarcoma patients included in our cohort had tumors which expressed β-hCG at diagnosis and post neoadjuvant chemotherapy, including 25% of patients who had β-hCG staining of over 50% post neoadjuvant chemotherapy. The patients who had post-treatment β-hCG staining greater than or equal to 50% of tumors cells had a statistically significant decreased 2 year PFS and trend towards worse OS. This is similar to previous studies correlating positive β-hCG staining with worse outcomes, but more specifically defines significance in the post-treatment setting. This provides a prognostic marker unique to the individual tumor post-neoadjuvant therapy and we speculate this may be a marker of chemoresistance or even a mechanism with upregulation of β-hCG. Further investigation is needed in the prospective setting with a larger study population and consideration of additional markers including anti-apoptotic proteins and tumorigenic cytokines to better understand the role of β-hCG expression in osteosarcoma tumors. Our cohort size was too small to evaluate the significance of change in β-hCG expression from the diagnostic specimen compared to the post-treatment tissue. Our findings correlating negative P16 expression in tumors to more unfavorable clinical outcomes are similar to previous reports [9.11] and confirm P16 as a potential prognostic marker at diagnosis.

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