Prognostic and Therapeutic Utility of Variably Expressed Cell Surface Receptors in Osteosarcoma

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Abstract

Background: Six cell surface receptors, human epidermal growth factor receptor (Her)-2, platelet-derived growth factor receptor (PDGFR)-β, insulin-like growth factor 1 receptor (IGF-1R), insulin receptor (IR), c-Met, and vascular endothelial growth factor receptor (VEGFR)-3, previously demonstrated variable expression across varying osteosarcoma (OS) cell lines. The current study sought to validate previous expression patterns and evaluate whether these receptors offer prognostic and therapeutic value. Methods: Patient-derived OS samples (n = 52) were labeled with antibodies to Her-2, PDGFR-β, IGF-1R, IR, c-Met, and VEGFR-3. Expression was characterized using flow cytometry. The geometric mean fluorescent intensity (geoMFI) for each receptor was calculated relative to a negative control. The event-free survival (EFS) and overall survival for patients with positive receptor expression were estimated by the Kaplan-Meier method. Differences in hazard for EFS event and overall survival event for patients with positive receptor expression were assessed using the log-rank test. Results: All 6 receptors were variably expressed in the majority of cell lines. None of the 6 receptors, were found to be significant predictors of EFS or overall survival. The sum total number of positive receptors per cell line also failed to predict EFS or overall survival. Conclusion: The six cell surface receptors demonstrated variable expression across the majority of patient-derived OS samples tested. While receptor expression did not provide prognostic value, their consistent expression makes them attractive targets for future therapeutic approaches.

Introduction

Osteosarcoma (OS) is the most common non-hematologic primary bone malignancy and the fifth most common primary malignancy among adolescents and young adults [1, 2]. The overall 5-year survival has plateaued at roughly 70% and has not improved in nearly four decades [2-5]. Multiple cooperative efforts including the recent EURAMOS-1 trial and studies by the European Osteosarcoma Intergroup [6, 7] have repeatedly demonstrated that intensifying conventional chemotherapy alone is futile, underscoring the need for novel approaches. There remains an ongoing interest in identifying OS biomarkers for prognostication and therapeutic targets.

The cell surface receptors expression pattern in OS was previously studied using patient-derived and standard OS cell lines [8]. Insulin-like growth factor receptor 2 (IGF2R) was consistently overexpressed across all cell lines evaluated, and further investigated as a potential novel therapeutic target using radioimmunotherapy

[9, 10]. A second group of receptors including Human epidermal growth factor receptor (HER)-2, platelet-derived growth factor receptor (PDGFR)-β, IGF-1R, insulin receptor (IR), c-Met, and vascular endothelial growth factor receptor (VEGFR)-3 were found to be variably expressed. Consideration was given as to whether expression across these receptors could yield either prognostic or therapeutic utility for subsets of OS tumors.

The purpose of this study was to address the following questions: (1) Does the variable pattern of receptor expression corroborate previously reported findings? (2) Does the pattern of surface receptor expression provide prognostic utility?

Materials & Methods

Cell lines

Ninety-nine OS cells lines were obtained from the Children's Oncology Group (COG) biorepository. All samples originated from patients with high-grade localized OS and were banked following informed written patient consent and Institutional Review Board (IRB) approval. Clinical outcomes for each sample were blinded to the investigators and only associated at the time of analysis. Of these 99 patients, 50 patients survived, and 49 patients had died. Eighteen samples did not grow in culture, leaving 81 samples available for analysis. Fifty-two samples yielded a sufficient number of cells for data analysis. Forty-eight patients had survival data. One patient had 2 observations that were treated as independent observations. The survival analysis was performed using these 49 observations.

Flow Cytometry Analysis

Cells were thawed and centrifuged to remove their freezing medium. After re-suspension with MEM-α media and 10% fetal bovine serum, cells were counted to determine the number of live cells per sample. As close to 1 million live cells were stained and assessed using flow cytometry.

Cell staining was done using commercially available anti-human antibodies to Her-2, IGF-1R, IR, VEGFR-3, c-MET, and PDGFR- β receptors in accordance with manufacture instructions (Table 1). Each antibody was conjugated to one of three fluorophores – phycoerythrin (PE), fluorescein-isothiocyanate (FITC) or allophycocyanin (APC) (Table 1). Control tubes were prepared using isotype-matched antibodies.

Flow cytometry analysis was performed using a Becton Dickinson LSRII digital bench top flow cytometer (Becton Dickinson, Mountain View, CA). To gate for single live cells, standard forward and side scatter gating protocols with DAPI staining, were employed as follows: FSC-A/SSC-A, FSC-A/FSC-H, SSC-A/SSC-H, SSC-A/DAPI. A minimum of 1,000 single live cells was required for analysis. Data was analyzed using FlowJo software (BD Biosciences).

Receptor Characterization

Receptor expression was plotted across all assayed cell lines, and expressed relative to a negative control. The negative controls used for Her-2-PE and PDGFR- β -PE were their respective isotype-matched controls, while the C243 cell line was used as the negative control for IGF-1R-FITC, IR-FITC, c-Met-APC, and VEGFR3-APC. Cell line C243 yielded no meaningful expression for these four receptors, and statistically deemed to be a robust negative control.

Limited samples precluded the use of Fluorescent Minus One (FMO) tubes; however, the C243 line was felt to provide a better negative control, accounting for any auto-fluorescence. Additional negative control Molt4 cell line was used as validation for Her-2 receptor characterization. For each cell line, triplicate samples were analyzed in order to diminish the likelihood that the results were due to a technical error. The geometric mean fluorescent intensity (geoMFI) and negative control geoMFI for each receptor across all cell lines were calculated.

The geoMFI for each receptor was recorded and expressed relative to the respective negative control as a fold change and defined as ($\text{geoMFI}_{\text{positive}}/\text{geoMFI}_{\text{negative}}$). A receptor was considered positive if the fold change

in geoMFI was above the 50^{th} percentile of cell lines. Binary variables were assigned for Her-2, PDGFR- β, IGF-1R, IR, cMet, and VEGFR3 in every cell line, indicating their presence or absence.

Statistical Analysis

For each COG cell line, seven variables were analyzed. The first six were binary variables representing the presence or absence of: Her-2, PDGFR- β, IGF-1R, IR, c-Met, and VEGFR-3. The seventh variable was the total number of positive receptors for a given cell line.

Each demographic characteristic of interest plus the COG study that was the source of follow-up data was checked for association with inclusion in the analytic population and each pair of characteristics was checked for association within the analytic population by the exact conditional test of proportions. Age at enrollment was checked in this manner as a categorical variable (0-9, [?]10 years) and also as a continuous variable using the t -test or a one-way analysis of variance (ANOVA), as appropriate.

The outcome in terms of event free survival (EFS) and overall survival were compared among the groups defined by the demographic variables. Event free survival was defined as days from enrollment to either an event (relapse, progression, or death) or to last contact. For EFS, patients were considered censored at last contact if they did not experience an event. Overall survival was defined as days from enrollment until either death or last contact. Osteosarcoma patients were considered censored at last contact if they were alive at that time.

The EFS and overall survival for the 6 receptors, as well as the total number of positive receptors per cell line, were estimated by the Kaplan Meier method. Differences in hazard for EFS event and overall survival event for positive and negative patients in each of the 6 receptors, as well as the total number of positive receptors per cell line, were assessed using the log-rank test. Analyses were done in SAS9.4 using PROC LIFETEST and PROC FREQ.

Results

The geoMFI and negative control geoMFI for each cell line and the respective receptors are summarized in Table 2. In Figure 1, the fold change ($\text{geoMFI}_{\text{positive}}/\text{geoMFI}_{\text{negative}}$) for each receptor across all cell lines is shown. The majority of cell lines were found to express the receptors of interest relative to their negative control; Her-2 was expressed in 98% of cell lines, c-MET in 76.5%, IGF-1R in 76.5%, PDGFR- β in 70.5%, IR in 66.7%, and VEGFR-3 in 62.7%. Outliers included cell lines C232 and C331, which exhibited no or very low expression for all 6 receptors. Her-2 and PDGFR- β expression demonstrated more variability across all cell lines, compared to IGF-1R, IR, VEGFR-3, and c-MET, which demonstrate less variability (Figures 1 & 2). The majority of cell lines were noted to be positive for 2 of 6 receptors, while only 3 cell lines demonstrated positive expression for all 6 receptors (Figure 3, Table 3).

Metastatic status at diagnosis ($p = 0.0192$) was found to be significantly associated with overall survival. Tables 4 and 5 summarize those patients with EFS and overall survival events with relation to the respective receptors expressed. Hazard ratio analyses for each of the 6 receptors and total number of positive receptors per cell line did not find them to be significant predictors of EFS or overall survival (Tables 6 & 7).

Discussion

In this study Her-2, PDGFR-β, IGF-1R, IR, c-Met, and VEGFR-3 were variably expressed, reaffirming previously reported results [8]. Hassan et al. reported a mean and standard deviation $\text{geoMFI}_{\text{positive}}/\text{geoMFI}_{\text{negative}}$ for the six receptors across all cell lines much different in absolute value than those reported in the current study. However when calculating the coefficient of variation to account for these differences, variable expression clearly exists across all receptors in both studies (Supplementary Table 1). Despite corroborating these initially reported findings, expression failed to achieve statistical significance in predicting EFS or overall survival.

The prognostic value of Her-2 in OS has been debated in the past and remains controversial. Akatsuka et al. analyzed immunohistochemical expression of Her-2 in 81 tumor samples from patients with non-metastatic

OS treated with surgery and chemotherapy [11]. They found that Her-2 over-expression was associated with both significantly increased EFS (72.2% vs. 45.6% at 5 years, p = 0.03) and overall survival (79.7% vs. 58.2% at 5 years, p = 0.03). Additionally, decreased levels of Her-2 increased the risk of adverse events and death (rate ratio: 2.24 and 2.54; 95% CI, 1.07-4.72 and 1.09-5.67, respectively). In contrast, Zhang et al. performed a meta-analysis evaluating the relationship between Her-2 expression and overall survival [12]. They identified 16 OS studies that provided survival outcomes and identified samples as being Her-2 positive or negative. Overexpression of Her-2 was associated with decreased overall survival in both biopsy and surgically removed specimens (HR = 2.07, 95% CI: 1.16-3.72, p = 0.014; and HR = 2.02, 95% CI: 1.10-3.71, P = 0.024). Finally, the COG conducted a large prospective study of 149 patients with newly diagnosed OS to determine the prognostic value of Her-2 [13]. They were unable to demonstrate that Her-2 status was associated with EFS or overall survival in patients with localized disease, concluding Her-2 expression was not prognostic.

Despite conflicting evidence regarding the prognostic utility of Her-2, its role as a therapeutic target has been pursued. *In vitro*studies by Long et al. investigated the role of Lapatinib, an inhibitor of Her-2 phosphorylation, in standard OS cell lines [14]. They found a dose- and time-dependent inhibition of cellular proliferation, higher apoptotic rates, and inhibition of migratory/invasive abilities. Rainusso et al. utilized Her-2-specific CAR T-cells to target tumor-initiating cells (TICs) in OS within an orthotopic xenograft model [15]. *In vivo* administration of the Her-2-specific T cells significantly reduced TICs, as evidenced by a reduction in sarcosphere forming efficiency in the explanted tumors. A phase II clinical trial, involving 96 patients with newly diagnosed metastatic OS, sought to determine the safety and feasibility of Trastuzumab as an adjunct to chemotherapy in patients whose tumors overexpressed Her-2 [16]. The 30-month EFS and overall survival for patients with Her-2 overexpression treated with chemotherapy and trastuzumab were 32% and 59%, respectively. Patients without Her-2 overexpression treated with chemotherapy alone demonstrated EFS and overall survival of 32% and 50%, respectively. These results failed to demonstrate significant improvement in survival by the addition of Trastuzumab. While Her-2 remains a feasible target, further investigation into its clinical value is needed.

There are numerous reports that have characterized the role of the IR/IGF-1R signaling pathway in the tumorigenesis and metastasis of various cancers [17, 18]. Li et al. showed that over-expression of IGF-1R promotes cellular proliferation, survival, and drug resistance, subsequently leading to OS metastasis [19]. Wang et al. compared expression levels of IGF-1R mRNA and proteins in 26 OS versus non-cancerous bone samples; both mRNA and proteins levels were found to be significantly higher within the OS samples [20]. Using 84 OS samples, IGF-1R expression was correlated to survival. High IGF-1R expression was associated with poorer survival, with multivariate Cox analyses demonstrating it to be an independent prognostic marker.

Both IR and IGF-1R have been investigated as therapeutic targets. A number of preclinical *in vitro* studies using OS cell lines have successfully suppressed cell proliferation, migration and invasion by inhibiting the IR/IGF-1R signaling pathway using miRNA, siRNA, and inhibitory antibodies [18, 21-25]. Kolb et al. used R1507, an anti-IGF-1R antibody, in OS xenograft tumor models to delay tumor growth in 4 of 6 OS xenografts with significant improvement in EFS [26]. Anderson et al. conducted a multi-institutional phase 2 clinical study using robatumumab in patients with relapsed OS and Ewing sarcoma [27]. In OS patients with resectable tumors the median overall survival was 20 months, while OS patients with unresectable tumors had a median overall survival of 8.2 months. The authors concluded that IGF-1R was targetable, though additional investigation was needed.

Platelet derived growth factor has been implicated in the tumorigenesis and metastasis of several solid tumors, and shown to portend a poor prognosis [28, 29]. Its role in the progression and prognosis of OS has been investigated as well [30, 31]. Kubo et al. examined surgical specimens from 54 OS patients, comparing the level of PDGF (-AA, -α, -BB, -β) receptor expression through immunohistochemistry to patient prognosis. They found PDGF-AA and PDGF-α receptors were correlated with inferior EFS ($p < 0.05$), while PDGF-BB and PDGF-β did not correlate to inferior EFS (p = 0.15). They also evaluated imatinib mesylate as a

therapeutic agent for OS. However, excessively high doses were required to achieve cytotoxicity and pathway inhibition, making this therapeutic approach unfeasible.

Imatinib has been utilized in both pre-clinical and early phase clinical studies. Yamaguchi et al. evaluated the *in vivo* anti-tumor effects of imatinib versus imatinib and doxorubicin in mice with heterotopically injected OS tumors. They demonstrated that combination therapy yielded synergistic effects, inhibiting cell proliferation [32]. The COG conducted a phase 2 clinical study looking at the effects of imatinib in children with refractory or relapsed solid tumors [33]. None of their OS patients demonstrated response according to Response Evaluation Criteria in Solid Tumors (RECIST). They were unsuccessful in showing imatinib was an effective, single-agent treatment.

The MET signaling pathway has been well described and implicated in the epithelial-mesenchymal transition (EMT) of tumor cells [34]. Thus, c-Met has been investigated as a potential target to inhibit tumor progression and metastasis. *In vitro* studies utilizing miRNA to inhibit c-Met have been successful in preventing cell proliferation, migration and invasion [35, 36]. Cabozantinib, an inhibitor of c-Met, has been investigated both in pre-clinical and clinical settings. Fioramonti et al. showed that cabozantinib decreased OS cell proliferation and migration through its effects on OS cells and their microenvironment [37]. The French Sarcoma Group conducted a phase 2 combined clinical trial using cabozantinib in patients with advanced Ewing sarcoma or OS, to assess efficacy both histologically and radiographically [38]. Five of 42 patients (12%; 95% CI 4-26) with OS had objective responses by 6 months; 14 of 42 patients (33%; 95% CI 20-50) had 6-month non-progression. They concluded that cabozantinib was well tolerated and demonstrated anti-tumor effects, warranting further investigation.

Vascular endothelial growth factor has been extensively reported in the literature to be associated with poor prognosis in OS due to its promotion of angiogenesis and metastasis [39-42]. Similar to the previously discussed receptors, VEGF has been investigated as a therapeutic target both *in vitro* and clinically. Studies have utilized a variety of miRNAs to inhibit the VEGF pathway and successfully suppressed cell proliferation, invasion and angiogenesis in standard OS cell lines [43-45].

Grignani et al. conducted a non-randomized, phase 2 clinical trial assessing the efficacy of sorafenib, an anti-VEGF antibody, and everolimus in patients with unresectable high-grade OS that had progressed despite standard chemotherapy treatment [46]. Of the 38 patients enrolled, 17 were progression free at six months (45%; 95% CI 28-61). They failed to demonstrate that treatment with sorafenib and everolimus improved disease progression at six months, despite having a small proportion of patients who were progression free. Navid et al. completed phase 2 clinical trials to evaluate the role of bevacizumab as an adjunct to standard OS treatment [47]. Thirty-one patients with localized OS received bevacizumab and chemotherapy both pre- and post-operatively. The estimated 4-year EFS and overall survival rate were 57.5 +/- 10% and 83.4 +/- 7.8 %, respectively. They concluded that while bevacizumab is a tolerable adjunctive therapy, the histologic tumor responses and EFS did not support further investigation..

It is valuable to distinguish inhibiting a receptor and its associated pathway from using a receptor as a means of targeting the expressing cell. The former approach needs the pathway to be functional and critical if the therapeutic measure is to have an impact. The latter approach is pathway-independent and uses the receptor solely for directing the therapeutic agent to the cell of interest. Targeting can be accomplished using a variety of means, including radioimmunotherapy and antibody-drug conjugates, both of which have been of interest in the setting of OS. IGF2-R targeted radioimmunotherapy has been explored in the preclinical arena and DS-8201 (trastuzumab deruxtecan), a HER-2 targeting antibody-drug conjugate, is currently being pursued by the COG. While uniquely expressed receptors or receptor patterns are ideal, consistent expression and overexpression may offer targeting opportunities, independent of associated intracellular pathways. A comprehensive understanding of a given osteosarcoma's surfaceome may prove increasingly useful.

In this study, we validated the variable expression of six cell surface receptors across a wide panel of patient-derived OS samples. We also sought to establish what prognostic value these receptors could offer. While our findings corroborate previous surface receptor expression results, they do not appear to offer obvious

prognostic utility. Nevertheless, the frequent expression of most receptors across most tumors raises the possibility that one or more could serve as a therapeutic target.

This study is limited by several factors. The experimental environment does not adequately recapitulate the human experience. *In vitro*tumors do not entirely reflect the *in vivo* state. Moreover, receptor expression was tested at a single point in time, assuming that expression is stable over time. We utilized the 50^{th}percentile as a threshold for positivity, recognizing that varying this cutoff could impact statistical results. Lastly, we analyzed a small sample from a larger tumor that is known to be genomically heterogeneous. Findings may be limited by sampling error, yielding results that may not be representative of the whole tumor.

In summary, although the evaluated six cell surface receptors fail to provide prognostic utility, they demonstrated variable expression across a panel of patient-derived OS samples. This finding, taken together with many prior publications, highlights the possibility of using one or more of these receptors in a targeted therapeutic manner. Osteosarcoma's genomic variability and high mutational burden makes it unlikely that a single treatment will adequately address all relapsed, metastatic, and/or chemo-resistant cases. One, or a combination, of the receptors discussed may prove useful in future targeted approaches and further investigation into such strategies is warranted.

References

1. Mirabello, L., R.J. Troisi, and S.A. Savage, *International osteosarcoma incidence patterns in children and adolescents, middle ages and elderly persons.* Int J Cancer, 2009. **125** (1): p. 229-34.

2. Kaatsch, P., *Epidemiology of childhood cancer.* Cancer Treat Rev, 2010. **36** (4): p. 277-85.

3. Mirabello, L., R.J. Troisi, and S.A. Savage, *Osteosarcoma incidence and survival rates from 1973 to 2004: data from the Surveillance, Epidemiology, and End Results Program.* Cancer, 2009.**115** (7): p. 1531-43.

4. Ferrari, S., et al., *Neoadjuvant chemotherapy with high-dose Ifosfamide, high-dose methotrexate, cisplatin, and doxorubicin for patients with localized osteosarcoma of the extremity: a joint study by the Italian and Scandinavian Sarcoma Groups.* J Clin Oncol, 2005.**23** (34): p. 8845-52.

5. Meyers, P.A., et al., *Osteosarcoma: a randomized, prospective trial of the addition of ifosfamide and/or muramyl tripeptide to cisplatin, doxorubicin, and high-dose methotrexate.* J Clin Oncol, 2005.**23** (9): p. 2004-11.

6. Whelan, J.S., et al., *EURAMOS-1, an international randomised study for osteosarcoma: results from pre-randomisation treatment.* Ann Oncol, 2015. **26** (2): p. 407-14.

7. Smeland, S., et al., *Survival and prognosis with osteosarcoma: outcomes in more than 2000 patients in the EURAMOS-1 (European and American Osteosarcoma Study) cohort.* Eur J Cancer, 2019. **109** : p. 36-50.

8. Hassan, S.E., et al., *Cell surface receptor expression patterns in osteosarcoma.* Cancer, 2012. **118** (3): p. 740-9.

9. Geller, D.S., et al., *Targeted therapy of osteosarcoma with radiolabeled monoclonal antibody to an insulin-like growth factor-2 receptor (IGF2R).* Nucl Med Biol, 2016. **43** (12): p. 812-817.

10. Karkare, S., et al., *Detection and targeting insulin growth factor receptor type 2 (IGF2R) in osteosarcoma PDX in mouse models and in canine osteosarcoma tumors.* Sci Rep, 2019. **9** (1): p. 11476.

11. Akatsuka, T., et al., *ErbB2 expression is correlated with increased survival of patients with osteosarcoma.* Cancer, 2002.**94** (5): p. 1397-404.

12. Zhang, Q., et al., *HER-2 expression in biopsy and surgical specimen on prognosis of osteosarcoma: A systematic review and meta-analysis of 16 studies.* Medicine (Baltimore), 2016.**95** (23): p. e3661.

13. Gorlick, S., et al., *HER-2 expression is not prognostic in osteosarcoma; a Children's Oncology Group prospective biology study.*Pediatr Blood Cancer, 2014. **61** (9): p. 1558-64.

14. Long, X.H., et al., *Lapatinib alters the malignant phenotype of osteosarcoma cells via downregulation of the activity of the HER2-PI3K/AKT-FASN axis in vitro.* Oncol Rep, 2014. **31** (1): p. 328-34.

15. Rainusso, N., et al., *Immunotherapy targeting HER2 with genetically modified T cells eliminates tumor-initiating cells in osteosarcoma.* Cancer Gene Ther, 2012. **19** (3): p. 212-7.

16. Ebb, D., et al., *Phase II trial of trastuzumab in combination with cytotoxic chemotherapy for treatment of metastatic osteosarcoma with human epidermal growth factor receptor 2 overexpression: a report from the children's oncology group.* J Clin Oncol, 2012.**30** (20): p. 2545-51.

17. Pollak, M., *The insulin and insulin-like growth factor receptor family in neoplasia: an update.* Nat Rev Cancer, 2012.**12** (3): p. 159-69.

18. Buck, E., et al., *Compensatory insulin receptor (IR) activation on inhibition of insulin-like growth factor-1 receptor (IGF-1R): rationale for cotargeting IGF-1R and IR in cancer.* Mol Cancer Ther, 2010. **9** (10): p. 2652-64.

19. Li, Y.S., et al., *The possible role of insulin-like growth factor-1 in osteosarcoma.* Curr Probl Cancer, 2019. **43** (3): p. 228-235.

20. Wang, Y.H., et al., *Increased expression of insulin-like growth factor-1 receptor is correlated with tumor metastasis and prognosis in patients with osteosarcoma.* J Surg Oncol, 2012.**105** (3): p. 235-43.

21. Zheng, S.W., et al., *Leptocarpin Suppresses Proliferation, Migration, and Invasion of Human Osteosarcoma by Targeting Type-1 Insulin-Like Growth Factor Receptor (IGF-1R).* Med Sci Monit, 2017.**23** : p. 4132-4140.

22. Chen, G., et al., *MicroRNA-133a Inhibits Osteosarcoma Cells Proliferation and Invasion via Targeting IGF-1R.* Cell Physiol Biochem, 2016. **38** (2): p. 598-608.

23. Liu, Y., et al., *MiR-100 Inhibits Osteosarcoma Cell Proliferation, Migration, and Invasion and Enhances Chemosensitivity by Targeting IGFIR.* Technol Cancer Res Treat, 2016. **15** (5): p. NP40-8.

24. Kuijjer, M.L., et al., *IR/IGF1R signaling as potential target for treatment of high-grade osteosarcoma.* BMC Cancer, 2013.**13** : p. 245.

25. Avnet, S., et al., *Insulin receptor isoform A and insulin-like growth factor II as additional treatment targets in human osteosarcoma.*Cancer Res, 2009. **69** (6): p. 2443-52.

26. Kolb, E.A., et al., *R1507, a fully human monoclonal antibody targeting IGF-1R, is effective alone and in combination with rapamycin in inhibiting growth of osteosarcoma xenografts.* Pediatr Blood Cancer, 2010. **55** (1): p. 67-75.

27. Anderson, P.M., et al., *A phase II study of clinical activity of SCH 717454 (robatumumab) in patients with relapsed osteosarcoma and Ewing sarcoma.* Pediatr Blood Cancer, 2016. **63** (10): p. 1761-70.

28. Uren, A., et al., *Beta-platelet-derived growth factor receptor mediates motility and growth of Ewing's sarcoma cells.* Oncogene, 2003.**22** (15): p. 2334-42.

29. Sulzbacher, I., et al., *Platelet-derived growth factor-alpha receptor expression supports the growth of conventional chondrosarcoma and is associated with adverse outcome.* Am J Surg Pathol, 2001.**25** (12): p. 1520-7.

30. Sulzbacher, I., et al., *Platelet-derived growth factor-AA and -alpha receptor expression suggests an autocrine and/or paracrine loop in osteosarcoma.* Mod Pathol, 2000. **13** (6): p. 632-7.

31. Kubo, T., et al., *Platelet-derived growth factor receptor as a prognostic marker and a therapeutic target for imatinib mesylate therapy in osteosarcoma.* Cancer, 2008. **112** (10): p. 2119-29.

32. Yamaguchi, S.I., et al., *Synergistic antiproliferative effect of imatinib and adriamycin in platelet-derived growth factor receptor-expressing osteosarcoma cells.* Cancer Sci, 2015.**106** (7): p. 875-82.

33. Bond, M., et al., *A phase II study of imatinib mesylate in children with refractory or relapsed solid tumors: a Children's Oncology Group study.* Pediatr Blood Cancer, 2008. **50** (2): p. 254-8.

34. Jeon, H.M. and J. Lee, *MET: roles in epithelial-mesenchymal transition and cancer stemness.* Ann Transl Med, 2017. **5** (1): p. 5.

35. Li, Q., et al., *MicroRNA-449b-5p Suppresses Proliferation, Migration, and Invasion of Osteosarcoma by Targeting c-Met.* Med Sci Monit, 2019. **25** : p. 6236-6243.

36. Xie, W., et al., *MicroRNA-876-5p inhibits cell proliferation, migration and invasion by targeting c-Met in osteosarcoma.* J Cell Mol Med, 2019. **23** (5): p. 3293-3301.

37. Fioramonti, M., et al., *Cabozantinib Affects Osteosarcoma Growth Through A Direct Effect On Tumor Cells and Modifications In Bone Microenvironment.* Sci Rep, 2018. **8** (1): p. 4177.

38. Italiano, A., et al., *Cabozantinib in patients with advanced Ewing sarcoma or osteosarcoma (CABONE): a multicentre, single-arm, phase 2 trial.* Lancet Oncol, 2020. **21** (3): p. 446-455.

39. Abdullah, S.E. and R. Perez-Soler, *Mechanisms of resistance to vascular endothelial growth factor blockade.* Cancer, 2012.**118** (14): p. 3455-67.

40. Liu, Y., et al., *High expression levels of Cyr61 and VEGF are associated with poor prognosis in osteosarcoma.* Pathol Res Pract, 2017.**213** (8): p. 895-899.

41. Mohamed, F.E.A., E.Z.I. Khalil, and N.D.M. Toni, *Caveolin-1 Expression Together with VEGF can be a Predictor for Lung Metastasis and Poor Prognosis in Osteosarcoma.* Pathol Oncol Res, 2019.

42. Han, G., et al., *Effects of vascular endothelial growth factor expression on pathological characteristics and prognosis of osteosarcoma.* Clin Exp Med, 2016. **16** (4): p. 577-584.

43. Lv, T., et al., *miR-503 is down-regulated in osteosarcoma and suppressed MG63 proliferation and invasion by targeting VEGFA/Rictor.*Cancer Biomark, 2018. **23** (3): p. 315-322.

44. Yan, T., et al., *MicroRNA944 targets vascular endothelial growth factor to inhibit cell proliferation and invasion in osteosarcoma.* Mol Med Rep, 2018. **18** (6): p. 5221-5228.

45. Zhang, L., et al., *MicroRNA-134 inhibits osteosarcoma angiogenesis and proliferation by targeting the VEGFA/VEGFR1 pathway.*FEBS J, 2018. **285** (7): p. 1359-1371.

46. Grignani, G., et al., *Sorafenib and everolimus for patients with unresectable high-grade osteosarcoma progressing after standard treatment: a non-randomised phase 2 clinical trial.* Lancet Oncol, 2015.**16** (1): p. 98-107.

47. Navid, F., et al., *A phase II trial evaluating the feasibility of adding bevacizumab to standard osteosarcoma therapy.* Int J Cancer, 2017. **141** (7): p. 1469-1477.

Legend

Table 1. Summary of Antibodies and Their Respective Isotype Antibodies (Autofluorescence)

Table 2. Raw Flow Cytometry Expression Data expressed in Geometric Mean Fluorescent Intensity (MFI).

Table 3. Cell lines that are positive or negative for each receptor.

Table 4. Number of Event Free Survival (EFS) events per patient with respect to positive or negative receptors

Table 5. Number of Overall Survival events per patient with respect to positive or negative receptors

Table 6. Hazard ratio analysis for EFS per cell surface receptor

Table 7. Hazard ratio analysis for overall survival per cell surface receptor

Figure 1. Fold change in receptor expression defined as relative increase in expression relative to the corresponding negative control

Figure 2. Receptor expression patterns assigned by geometric mean fluorescent intensity (A) low expression pattern group (B) medium expression pattern group (C) high expression pattern group

Figure 3. Total number of positive receptors per cell line using the 50^{th} percentile as a cutoff for positivity.

Supplementary Table 1. Comparison between Hassan et al. and the current study, demonstrating mean $\text{geoMFI}_{\text{positive}}/\text{geoMFI}_{\text{negative}}$ and coefficient of variation (defined as standard deviation/mean) across all six surface receptors.

Table 1. Summary of Antibodies and Their Respective Isotype Antibodies (Autofluorescence)

Receptor Antibody (Fluorophore)	Vendor/Catalog No.	Isotype Antibody	Positive Control
Monoclonal anti-human HER-2/neu (PE)	BD/340552	mouse IgG1/Neu24.7	MCF7 (ATCC/HTB-22)
Monoclonal anti-human CD140b/PDGFR- β (PE)	R&D/FAB1263P	mouse IgG1/PR7212	Hs 697.Sp (ATCC/CRL-7433)
Monoclonal anti-human IGF-1R (FITC)	R&D/FAB391F	mouse IgG1/33255	MCF7 ATCC/HTB-22)
Polyclonal anti-human IR (AF488)	R&D/FAB1544G	goat IgG/NP_0010732 85	monocytes (periph blood sample)
Monoclonal anti-human HGFR/c-MET (APC)	R&D/FAB3582A	mouse IgG1/95106	monocytes (periph blood sample)
Monoclonal anti-human VEGFR-3 (APC)	R&D/FAB3492A	mouse IgG1/54733	monocytes (periph blood sample)

Table 2. Raw Flow Cytometry Expression Data expressed in Geometric Mean Fluorescent Intensity (MFI).

Cell Line	HER-2		PDGFR-β		IGF-1R		IR		c-MET		VEGFR-3	
	GM	Negative GM	GM	Negative GM	GM	Negative GM	GM	Negative GM	GM	Negative GM	GM	Negative GM
C194	3813	274	2272	274	366	340	417	431	1334	808	444	507
C204	2436	187	652	187	195	351	212	442	1688	830	208	520
C205	10890	1473	4475	1473	982	351	1061	442	5352	830	1403	520
C209	4307	569	4012	569	338	351	474	442	3107	830	466	520
C219	3920	376	4741	376	575	351	556	442	1802	830	864	520
C231	4281	722	2743	722	461	351	590	442	1031	830	618	520
C232	2925	237	3028	237	431	351	446	442	828	830	513	520
C233	3968	128	2755	128	404	351	392	442	1523	830	664	520
C238	3391	301	3653	301	474	351	522	442	1112	830	690	520
C243	1731	231	2370	231	349	271	437	271	821	529	517	529
C244	2446	236	5676	236	368	351	498	442	726	830	547	520
C251	7002	1276	10191	1276	1260	351	1378	442	3170	830	2224	520
C253	4378	425	7153	425	445	351	685	442	3038	830	764	520
C256	2345	276	941	276	307	351	349	442	2934	830	361	520
C281	4661	552	3198	552	734	351	766	442	2775	830	1046	520
C282	6446	553	12818	553	749	351	613	442	957	830	891	520
C287	2225	377	5033	377	469	351	498	442	1170	830	581	520
C290	3532	375	4540	375	464	351	426	442	904	830	553	520
C291	3997	445	4867	445	398	351	571	442	3957	830	726	520
C293	3160	375	1904	375	526	351	577	442	835	830	436	520
C297	4020	394	1401	394	396	351	453	442	2420	830	464	520
C299	4357	263	2035	263	379	351	355	442	1176	830	378	520
C300	3125	337	729	337	351	351	409	442	3047	830	416	520
C301	2451	129	1372	129	177	351	210	442	315	830	177	520
C307	5177	569	1674	569	685	351	566	442	3677	830	725	520
C311	2682	305	4561	305	353	351	443	442	844	830	419	520
C314	1483	198	1901	198	289	351	326	442	752	830	223	520
C315	3443	521	4091	521	604	351	606	442	1570	830	621	520
C323	8697	859	5369	859	988	351	843	442	2534	830	1012	520
C326	4273	304	4210	304	391	351	363	442	812	830	366	520
C327	7755	882	13316	882	586	351	704	442	1196	830	1000	520
C331	2161	353	1285	353	360	351	413	442	1079	830	406	520
C334	3356	487	3298	487	490	351	534	442	993	830	558	520
C337	2920	386	1248	386	475	351	486	442	1845	830	567	520
C338	5374	842	5284	842	616	351	724	442	903	830	690	520
C340	5275	1462	12751	1462	865	351	900	442	1915	830	1491	520
C342	3441	269	5408	269	580	351	601	442	830	442	237	520
C346	2148	348	1177	348	331	351	398	442	1146	830	395	520
C349	5244	610	6503	610	739	351	816	442	1816	830	836	520
C353	3640	605	2066	605	740	351	900	442	994	830	659	520
C360	2955	430	1230	430	476	351	537	442	1358	830	560	520
C365	6878	846	9037	846	546	351	607	442	1902	830	737	520
C366	4736	612	9757	612	402	351	468	442	990	830	627	520
C368	5245	137	6416	337	228	351	272	442	531	830	226	520
C370	10605	637	23437	637	442	351	733	442	6160	830	701	520
C371	7012	379	10890	379	271	351	455	442	1134	830	419	520
C373	10606	548	44596	548	462	351	769	442	1356	830	551	520
C374	13516	1229	26729	1229	843	351	1076	442	2565	830	1028	520
C375	2256	435	5113	435	639	351	695	442	593	830	417	520
C377	3154	252	4001	252	319	351	380	442	670	830	264	520
C379	4040	384	5361	384	447	351	480	442	1019	830	393	520
C396	18102	1431	22774	1431	966	351	1148	442	8388	830	1299	520

Table 3. Cell lines that are positive or negative for each receptor.

Cell line	HER-2	PDGFR-β	IGF-1R	IR	c-MET	VEGFR-3	Total
C194	positive	negative	negative	negative	positive	negative	2
C204	positive	negative	negative	negative	positive	negative	2
C205	negative	negative	positive	positive	positive	positive	4
C209	negative	negative	negative	negative	positive	negative	1
C219	positive	positive	positive	positive	positive	positive	6
C231	negative	negative	negative	positive	negative	positive	2
C232	positive	positive	negative	negative	negative	negative	2
C233	positive	positive	negative	negative	positive	positive	4
C238	positive	positive	positive	negative	negative	positive	4
C243	negative	negative	negative	negative	negative	negative	0
C244	positive	positive	negative	negative	negative	negative	2
C251	negative	negative	positive	positive	positive	positive	4
C253	positive	positive	negative	positive	positive	positive	5
C256	negative	negative	negative	negative	positive	negative	1
C281	negative	negative	positive	positive	positive	positive	4
C282	positive	positive	positive	positive	negative	positive	5
C287	negative	positive	positive	negative	negative	positive	3
C290	positive	positive	positive	negative	negative	negative	3
C291	negative	positive	negative	positive	positive	positive	4
C293	negative	negative	positive	positive	negative	negative	2
C297	positive	negative	negative	negative	positive	negative	2
C299	positive	negative	negative	negative	negative	negative	1
C300	positive	negative	negative	negative	positive	negative	2
C301	positive	negative	negative	negative	negative	negative	1
C307	positive	negative	positive	positive	positive	positive	5
C311	negative	positive	negative	negative	negative	negative	1
C314	negative	negative	negative	negative	negative	negative	0
C315	negative	negative	positive	positive	positive	positive	4
C323	positive	negative	positive	positive	positive	positive	5
C326	positive	positive	negative	negative	negative	negative	2
C327	negative	positive	positive	positive	negative	positive	4
C331	negative	negative	negative	negative	negative	negative	0
C334	negative	negative	positive	negative	negative	negative	1
C337	negative	negative	positive	negative	positive	positive	3
C338	negative	negative	positive	positive	negative	positive	3
C340	negative	negative	positive	positive	positive	positive	4
C342	positive	positive	positive	positive	positive	negative	5
C346	negative	negative	negative	negative	negative	negative	0
C349	negative	positive	positive	positive	positive	positive	5
C353	negative	negative	positive	positive	negative	positive	3
C360	negative	negative	positive	positive	negative	positive	4
C365	negative	positive	positive	positive	positive	positive	5
C366	negative	positive	negative	negative	negative	positive	2
C368	positive	positive	negative	negative	negative	negative	2
C370	positive	positive	negative	positive	positive	positive	5
C371	positive	positive	negative	negative	negative	negative	2
C373	positive	positive	positive	positive	positive	negative	5
C374	positive	positive	positive	positive	positive	positive	6
C375	negative	positive	positive	positive	negative	negative	3
C377	positive	positive	negative	negative	negative	negative	2
C379	positive	positive	negative	negative	negative	negative	2
C396	positive	positive	positive	positive	positive	positive	6
Total	26	26	26	25	26	26	

Table 4. Number of Event Free Survival (EFS) events per patient with respect to positive or negative receptors

	EFS Event		
Analysis Variables	**No**	**Yes**	**Total**
Her2			
No	8	16	24
Yes	9	15	24
PDGFRB			
No	7	17	24
Yes	10	14	24
IGFIR			
No	6	17	23
Yes	11	14	25
IR			
No	8	17	25
Yes	9	14	23
cMet			
No	10	14	24
Yes	7	17	24
VEGFR3			
No	6	18	24
Yes	11	13	24
Total Positive			
0	1	2	3
1	1	5	6
2+	15	24	39

Table 5. Number of Overall Survival events per patient with respect to positive or negative receptors

Analysis Variables	Life Status		Total
	Alive	Dead	
Her2			
No	13	11	24
Yes	17	7	24
PDGFRB			
No	13	11	24
Yes	17	7	24
IGFIR			
No	13	10	23
Yes	17	8	25
IR			
No	14	11	25
Yes	16	7	23
cMet			
No	16	8	24
Yes	14	10	24
VEGFR3			
No	14	10	24
Yes	16	8	24
Total Positive			
0	1	2	3
1	4	2	6
2+	25	14	39

Table 6. Hazard ratio analysis for EFS per cell surface receptor

Analysis Variable (Value Ratio)	Hazard Ratio	95% Confidence Interval for HR	*p*-value for HR ≠ 1
Her2 (Yes vs. No)	0.824	(0.405, 1.675)	0.5922
PDGFRB (Yes vs. No)	0.809	(0.392, 1.669)	0.5655
IGFIR (Yes vs. No)	0.795	(0.391, 1.615)	0.5256
IR (Yes vs. No)	1.179	(0.568, 2.445)	0.6591
cMet (Yes vs. No)	1.576	(0.772, 3.220)	0.2118
VEGFR3 (Yes vs. No)	0.900	(0.438, 1.846)	0.7728
Total Positive			
(1 vs. 0)	1.023	(0.197, 5.300)	0.9785
(2+ vs. 0)	1.062	(0.250, 4.511)	0.9355

Table 7. Hazard ratio analysis for overall survival per cell surface receptor

Analysis Variable (Value Ratio)	Hazard Ratio	95% Confidence Interval for HR	*p*-value for HR ≠ 1
Her2 (Yes vs. No)	0.545	(0.209, 1.415)	0.2123
PDGFRB (Yes vs. No)	0.638	(0.245, 1.662)	0.3574
IGFIR (Yes vs. No)	0.879	(0.345, 2.239)	0.7865
IR (Yes vs. No)	0.931	(0.355, 2.443)	0.8845
cMet (Yes vs. No)	1.735	(0.669, 4.499)	0.2571
VEGFR3 (Yes vs. No)	1.148	(0.447, 2.953)	0.7742
Total Positive			
(1 vs. 0)	0.472	(0.066, 3.356)	0.4531
(2+ vs. 0)	0.674	(0.152, 2.986)	0.6033

Figure 1. Fold change in receptor expression defined as relative increase in expression relative to the corresponding negative control

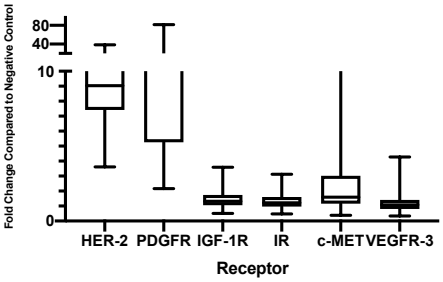


Figure 2. Receptor expression patterns assigned by geometric mean fluorescent intensity (A) low expression pattern group (B) medium expression pattern group (C) high expression pattern group

(A)

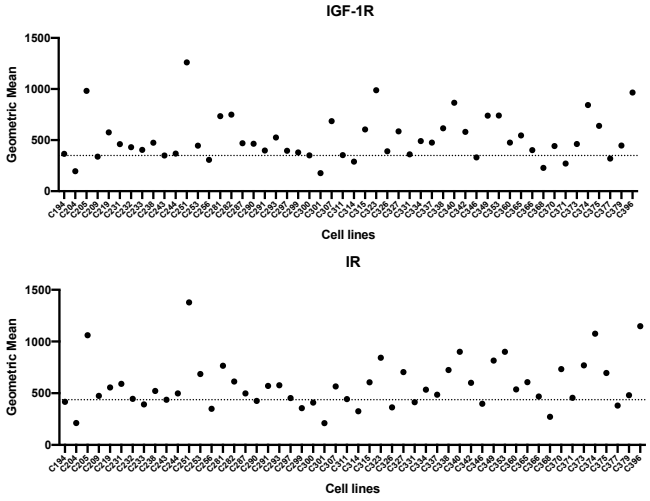
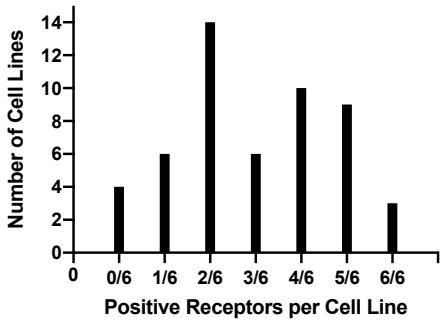


Figure 3. Total number of positive receptors per cell line using the 50th percentile as a cutoff for positivity.



Supplementary Table 1. Comparison between Hassan et al. and the current study, demonstrating mean $geoMFI_{positive}/geoMFI_{negative}$ and coefficient of variation (defined as standard deviation/mean) across all six surface receptors.

	Hassan et al.		Current Study	
	Mean $geoMFI_{positive}/geoMFI_{negative}$ (Std. Dev.)	**Coefficient of Variation**	**Mean $geoMFI_{positive}/geoMFI_{negative}$ (Std. Dev.)**	**Coefficient of Variation**
Her2	0.31 (0.18)	0.59	10.76 (6.06)	0.56
PDGFRB	3.42 (3.76)	1.09	12.56 (12.19)	0.97
IGF1R	0.26 (0.30)	1.19	1.47 (0.63)	0.43
IR	1.69 (1.85)	1.09	1.32 (0.54)	0.41
cMET	0.67 (0.59)	0.88	2.24 (1.81)	0.81
VEGFR3	0.43 (0.34)	0.79	1.23 (0.71)	0.57