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Research Article

Histological and Immunohistochemical Prognostic Factors in Paediatric Osteosarcoma - A Retrospective Study

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Abstract

Osteosarcoma (OS) is the most common primary malignant tumour of bone, excluding myeloma and is the third most common malignant disease in adolescence after leukaemia and lymphoma [1].

Keywords: *Osteosarcoma; Chemotherapy; Metastasis Free Survival*

Introduction

Osteosarcoma (OS) is the most common primary malignant tumour of bone, excluding myeloma and is the third most common malignant disease in adolescence after leukaemia and lymphoma [1]. It accounts for 20% of all primary malignant tumor [1].

Treatment of osteosarcoma typically includes preoperative chemotherapy (CT), surgical resection, and postoperative CT. Limb-salvage procedures with wide surgical margins are the mainstay of surgical intervention [2]. Early adjuvant treatment with pre- and post-surgical CT coupled with limb-sparing surgery has achieved a 5-year disease-free survival of approximately 60% in patients without pulmonary metastasis at presentation [3]. Although this represents a significant advance in the management of these patients, those who relapse often fail to respond to salvage therapy. Attempts to intensify therapy based on less than optimal histological response to preoperative chemotherapy have not improved outcome. Thus, there is a need for alternate adjuvant chemotherapy (NACT).

Besides clinical stage, there are no prognostic indicators with sufficient predictive value at the time of diagnosis. However, response of the tumour to CT which is assessed by histology can be considered as a significant predictive factor of outcome and is assessed after several weeks of therapy.

Malignant bone tumours represent 6 - 7% of all paediatric neoplasms [4]. OS accounts for 30% of bone tumors in the Regional Cancer Center, Trivandrum, India. Some of the biologic factors evaluated thus far as potential prognostic factors in OS are the expression of P-glycoprotein (P-gp) and erb-2 [5].

There is considerable debate about important prognostic factors in OS. Prognosis after OS recurrence is poor, with a less than 20% long-term survival rate. Saeter G., *et al.* [6] says that the most important prognostic factors in high grade OS appear to be presence of detectable metastasis at diagnosis, tumor volume, old age, sex, histologic response and possibly tumoral p-glycoprotein expression. In some studies HER2/erb2 expression in osteosarcoma has been shown to be associated with poor prognosis (poor histologic response to preoperative chemotherapy and decreased event free survival). A study by Fellenberg., *et al.* [7] Her-2/neu expression was demonstrated in 94% cases. Her-2/neu expression was significantly elevated in patients with poor response to chemotherapy. H Zhou., *et al.* [8] showed that cytoplasmic Her-2/neu expression is associated with a worse metastasis free survival(MFS) in patients with or without metastasis at the initial diagnosis and may be a distinguishing feature of a more aggressive tumour phenotype in OS.

Aim of the Study

1. To study the expression of P-glycoprotein and HER-2/neu protein in the pre-treatment biopsy specimen and correlate this with the preoperative chemotherapeutic response.

2. To assess histopathological and immunohistochemical prognostic factors of osteosarcomas that affect the survival.

Materials and Methods

The study was conducted at the Division of Pathology, Regional Cancer Centre, Thiruvananthapuram, India.

Subject selection

In this retrospective study 133 cases were included which were diagnosed as osteosarcoma during a period of eleven years from 1st January 2000 - 31st December 2010.Cases where no clinical details available were excluded. Immunohistochemistry (IHC) was assessed on the availability of tissue blocks which could be submitted for adequate sectioning. IHC analysis was done in 36 cases where the blocks were retrieved.

Details regarding post chemotherapy response were obtained from the case records. After chemotherapy the patient was subjected to surgery. The percentage of necrosis was assessed using Huvos grading system. This information was taken from the permanent case records. Based on this the patients were grouped into two categories according to previous studies as given below (Table 1).

> 90% necrosis	Good responders
< 90% necrosis	Bad responders

Table 1

Immunohistochemical analysis

Two 4 - 5 µm thick tissue sections were taken from representative block for each case. After heat induced antigen retrieval, immunohistochemical analysis was done by Supersensitive polymer HRP-IHC detection system. Appropriate positive and negative controls were included in all stains to ensure the quality and consistency of staining result. The positive control tissue used for P-gp was the human adrenal tissue and for Her-2/neu was breast carcinoma with strong Her-2 positivity. The results were interpreted manually by 2 observers.

The antibody against P-gp was monoclonal mouse anti human antibody, designed to localize the multi-drug resistance marker (p-glycoprotein) on formalin fixed paraffin embedded tissue sections. P-gp is a cell membrane protein of multi-drug resistant gene MDR-1. The expression was analysed as intensity of cytoplasmic or membrane positivity (Table 2). Further scoring was done based on percentage of cells showing positivity (Table 3) and interpreted as Mild, moderate and severe (1+, 2+, 3+). Any nuclear positivity was considered as negative.

Membrane/cytoplasmic positivity based on intensity	Score
Mild	1+
Moderate	2+
Strong	3+

Table 2: *Interpretation of p-glycoprotein expression.*

% of membrane positive cells	Score
No staining	0
1 - 25	1+
26 - 50	2+
51 - 75	3+
76 - 100	4+

Table 3: *Further scoring of p-glycoprotein.*

The antibody against Her-2 was rabbit monoclonal antibody, designed for specific localization of Her-2 on formalin-fixed paraffin embedded tissue sections. Her-2 is one of the four members of the ErbB receptor family of transmembrane receptor like tyrosine kinases. According to previous studies the expression was analysed as the percentage of membrane positive cells and results were interpreted as shown in table 4.

% of membrane positive cells	Score
No staining	0
< 30% cells with partial positivity	1+
> 30% cells with weak and moderate intensity staining complete membrane	2+
> 30% cells with strong intensity staining complete membrane	3+

Table 4: *Interpretation of Her -2/neu expression.*

Statistical analysis

Statistical Analysis was done using SPSS software v.17. Initially frequency distributions of all the study variables were taken. Overall survival was assessed by Kaplan-Meier method. Comparison of survival by various factors was assessed using Log Rank Test. Histological and immunohistochemical prognostic factors were assessed using Cox's proportional hazard regression model. Survival plots were made using Kaplan-Meier method.

Results

A total of 133 cases of osteosarcoma were included during a period of eleven years from 1st January 2000 - 31st December 2010. Cases were non-randomly selected based on the availability of permanent case records. Three cases were excluded as no details were available. Clinical details were collected from the case records. The cases were diagnosed by biopsy (n = 115) and FNAC (n = 18). Histological examination was done on cases where H&E slides were available (n = 64) and IHC was done for cases where tissue blocks (n = 36) were available. Maximum number of cases in our study belonged to 2nd decade i.e. 11-14 years. The age of the patients ranged between 0 - 14 years. 6 patients were below 5 years of age. Of the 133 cases, 71 were males and 62 were females. 72.3% cases presented within 3 months of onset of symptoms. Overall survival was 70.59% at 3-years and 30% at the end of 5-years. The median follow up time was 13 months (range 2 - 104 months). The median survival time was 19 months. Femur was the most common bone involved. Most of the lesion was lytic. Metaphysis of long bones were commonly involved. Alkaline phosphatase was the commonly elevated enzyme. Patients with elevated LDH showed less survival.

Osteoblastic OS was the commonest histologic subtype observed. Chondroblastic OS had better survival than other histologic subtypes. Her-2 was not expressed in any of the cases. All cases observed were high grade OS. No cases of surface osteosarcomas were there.

Number of mitosis/HPF and presence of necrosis in pre-treatment biopsy had no significance. Histologic response after NACT had no correlation with P-gp and Her-2 expression in pre-treatment biopsy sample. Histologic response following NACT had no significant correlation with survival.

The region of long bone where tumour occurred, intensity of p-glycoprotein positivity and presence of metastasis were statistically significant prognostic factors in osteosarcoma. Involvement of diaphysis had a poor survival. Overall survival was 70.59% at 3-years and 30% at the end of 5-years.

Study	IHC	Study findings
Gorlick., *et al*.	P-gp	No correlation between pgp status and % of OS tumour necrosis after induction CT.
Schwartz., *et al*.	P-gp	Tumour histological response to NACT did not correlate with pgp positivity. No significant difference in EFS or risk of death between patients with pgp positivity compared to pgp negative patients.
Serra., *et al*.	P-gp	p-gp positivity was found in 47 of 149 cases (32%) and was significantly associated with a higher incidence of relapse.
Baldani., *et al*.	P-gp	p-gp expression in tumor cells at diagnosis was significantly associated with a higher rate of systemic relapse ($p < 0.001$).
Baldani., *et al*.	P-gp	P-glycoprotein status ($P = 0.001$) and the extent of tumor necrosis after preoperative chemotherapy ($P=0.04$) were independent predictors of clinical outcome.
Wunder., *et al*.		no correlation between MDR1 gene expression and prognosis n = 129
Pakos., *et al*.		P-gp was not associated with the histologic response of patients with osteosarcoma to combination chemotherapy regimens. p-gp positivity was a strong correlate of more rapid disease progression
Our study	P-gp, Her-2	Survival was low in cases ($p = 0.02$) for P-gp positive cases. P-gp expression was not associated with the histologic response.

Table 5: *p-gp expression in OS.*

Study	IHC	Study findings
D.G. Thomas., *et al*.	Her-2	demonstrated an absence of HER 2/neu expression in OS (n = 55) and EWS (n = 11) and concluded that HER2/neu is not an important prognostic factor
Gorlick., *et al*.	Her2, p^{53}, pgp	53 samples studied. Her-2/erbB2 expression had significantly worse histologic response ($p = 0.03$) and decreased event free survival ($p = 0.05$).
Fellenberg., *et al*.		Her-2/neu expression was significantly elevated in patients with poor response to chemotherapy. Her-2/neu expression could be demonstrated in (n = 17) 94%cases.
Onda., *et al*.	erbB-2	In 42% of the OS expressed ErbB-2. Expression of ErbB-2 was strongly correlated with early pulmonary metastasis $P < 0.05$ and poor survival rate $(P < 0.01)$ for the patient.
Maitra., *et al*.	erbB-2	No cases showed (n = 21) HER-2/*neu* gene amplification by FISH AND IHC. Concluded that Her-2 amplification appeared to be an uncommon event in paediatric osteosarcomas.
Kilpatrick., *et al*.	HER-2	41 cases analysed. Cytoplasmic positivity was observed in most osteosarcomas, irrespective of histologic subtype/grade, and does not appear associated with response to preoperative chemotherapy or disease progression
H. Zhou., *et al*.	Her-2	Positive Her-2/*neu* expression (n = 25) was identified in 44% of the primary tumor samples and 58% of the metastatic pulmonary site samples.Her-2/neu expression is associated with a worse metastasis free survival may be a feature of a more aggressive phenotype in osteosarcoma. No significant relationship between Her-2/*neu* expression and the amount of tumor necrosis was identified.
Our study	Her-2, pgp	All cases were negative

Table 6: *HER-2 expression in OS.*

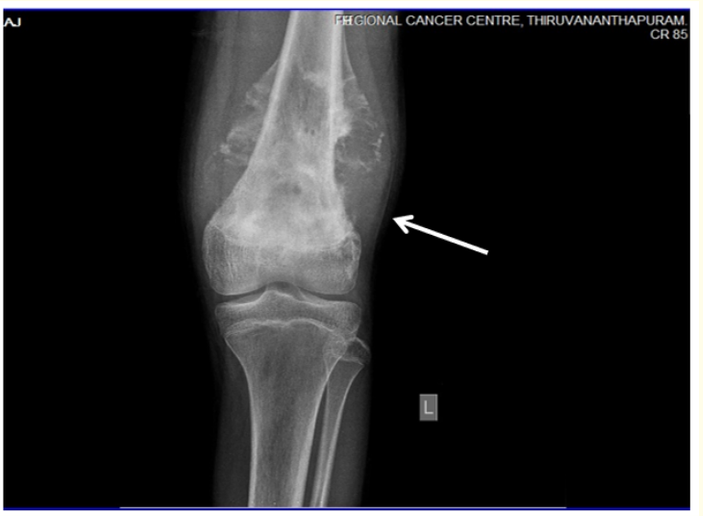

Figure 1: *Osteosarcoma -X-ray left femur showing periosteal reaction, Codman's triangle.*

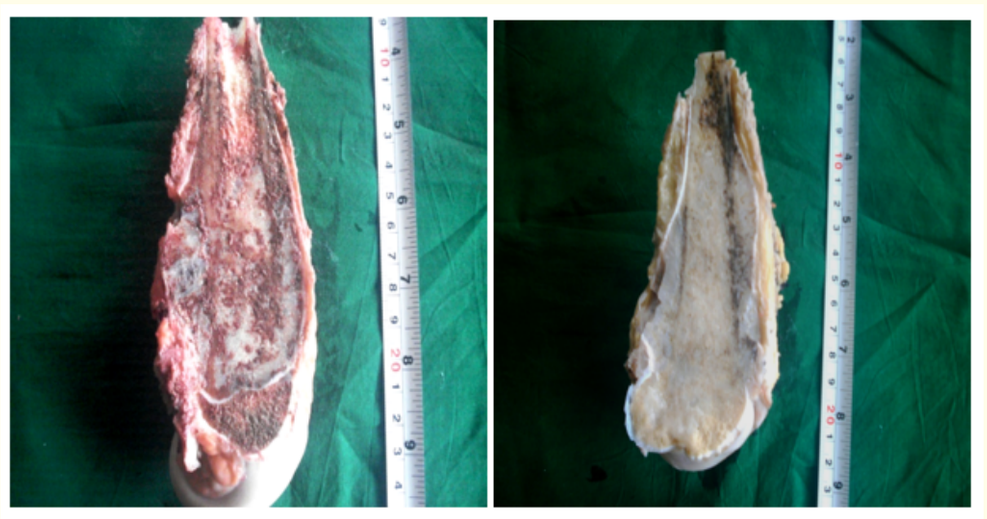

Figure 2: *Gross appearance of osteosarcoma.*

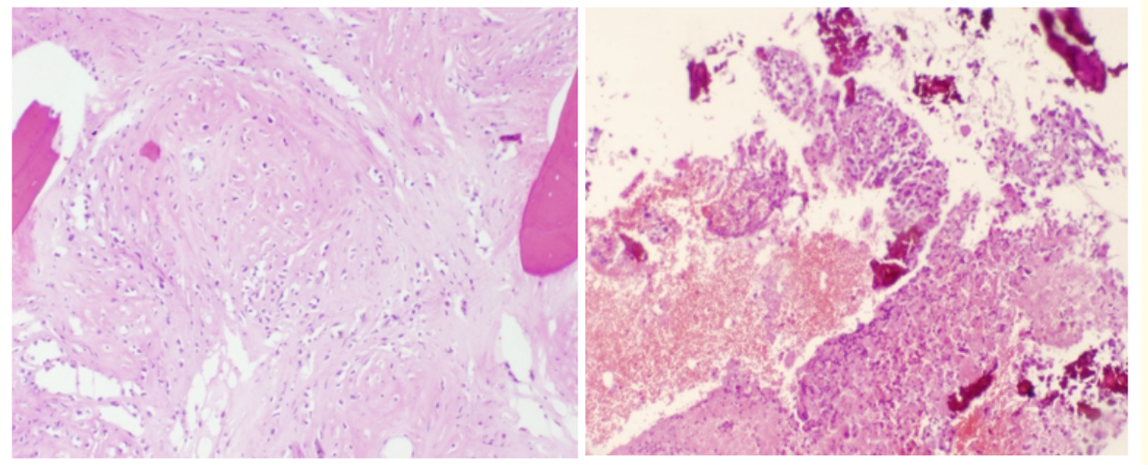

Figure 3: *Osteoblastic OS-permeating bony spicules pre CT biopsy of OS-necrosis.*

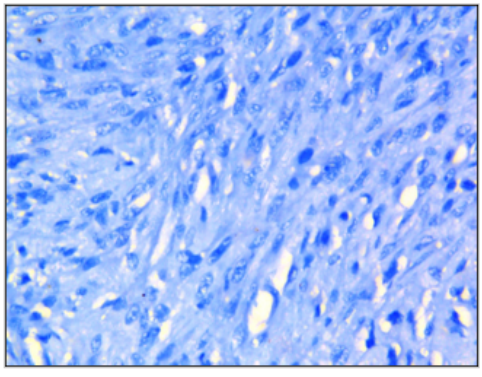

Figure 4: *OHer2 in OS-Negative.*

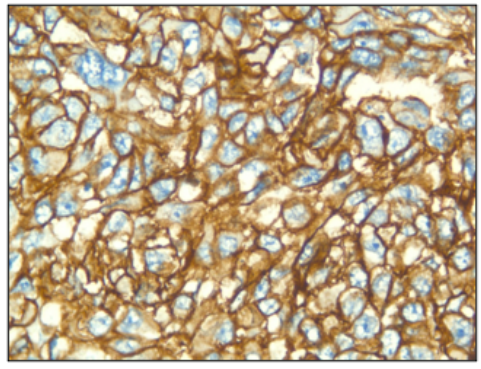

Figure 5: *Her2 in Breast Ca-+ve control.*

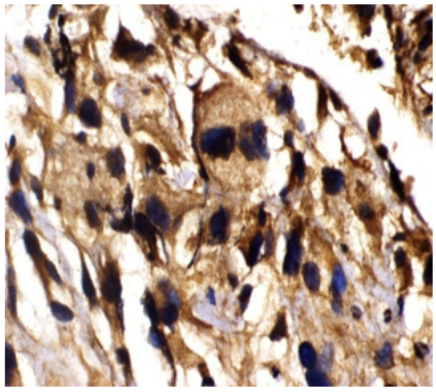

Figure 6: *P-Glycoprotein-Strong membrane and cytoplasmic positivity in OS.*

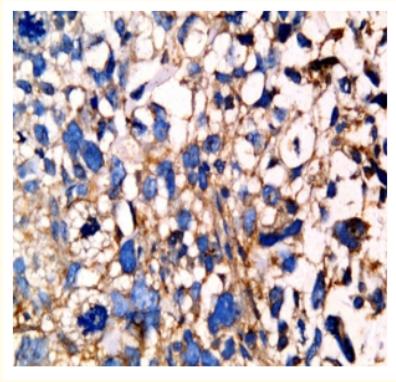

Figure 7: *P-Glycoprotein-Moderately +ve in OS.*

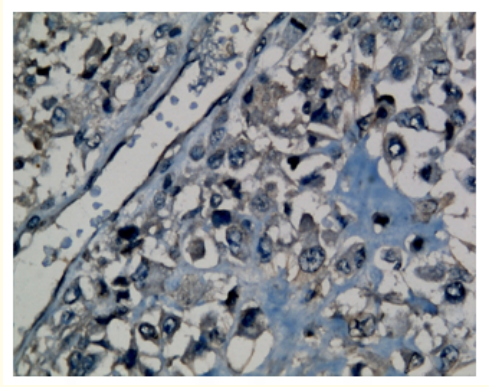

Figure 8: *P-glycoprotein weakly +ve in OS.*

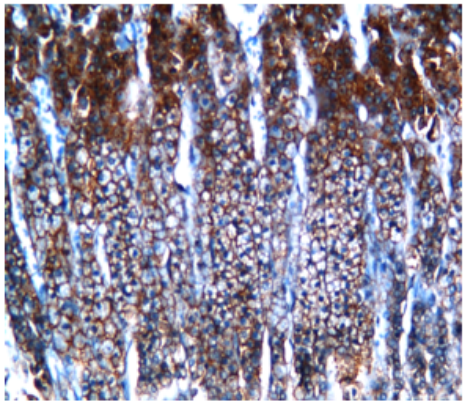

Figure 9: *P-glycoprotein in normal adrenal tissue- +ve control.*

Discussion

Osteosarcomas are highly aggressive mesenchymal tumour typically affecting long tubular bones. Treatment typically includes pre-operative chemotherapy, surgical resection and postoperative chemotherapy. At present, the ability to predict prognosis at diagnosis in osteosarcoma is limited. This study was designed to identify histological and immunohistochemical prognostic factors in paediatric cases of osteosarcoma and to determine the correlation between HER2/erb-2 and P-glycoprotein expression in pre-treatment biopsy samples and their correlation with histologic response to preoperative chemotherapy. The overall survival was also assessed. Ottavani., *et al.* [9] found that OS was extremely rare in patients younger than five years. In our study only 4.5% of cases were below 5 years.

The most frequent bones involved were long bones, with femur (58.13%) being the most common bone to be involved. This finding was similar to previous studies [5,8]. When long bones were involved it was the metaphysis which was the commonly involved region in majority of cases as in previous studies.

Histological examination revealed that osteoblastic type (42.2%) was the most common type. This was consistent with previous literature which showed 50% of osteoblastic type [10]. Other histological types were chondroblastic, fibroblastic and mixed types (consisting of previously mentioned subtypes in varying proportion). Rare types like telangiectatic, epithelioid, giant cell rich type and small cell OS were also seen. When the histological subtypes were correlated with survival, it was seen that the patients with chondroblastic subtype had better 5-year survival of 66.78% when compared to other subtypes like osteoblastic OS. Hauben EI., *et al.* [11,12] showed similar survival for the chondroblastic subtype. But there are studies which showed that histologic type had no correlation [13]. Klein., *et al.* [14] opined that this separation is largely artificial because there is no statistical difference in the survival of patients with high-grade tumors of these 3 histologic types and the treatment for all types is the same.

5-year survival for patients was low in cases where necrosis was observed in pre-treatment biopsy than in cases where it was not present. But this difference had no statistical correlation. Bjornsson., *et al*. [13,15] found that spontaneous necrosis was an adverse prognostic factor.

Expressions of P-Gp and Her-2/neu have been correlated with tumour regression and clinical outcome but reports are controversial. Hence we were prompted to analyse the immunohistochemical expression of P-glycoprotein and Her-2/neu in OS.

In the present study P-Gp expression in tumour cells of cases with osteosarcoma was done by Immunohistochemistry. We studied 36 cases where paraffin blocks were retrieved. 14 cases showed no positivity. Osteoid positivity was seen in two cases. Tumor cells showed positivity in 17 cases (50%). Both membrane and cytoplasmic expression were considered as positive. We found that any detectable over expression of P-Gp did not statistically correlate with histologic response ($p = 0.5$). This was consistent with previous studies by Meyers PA [16] that proposed that there is no correlation between P-Gp expression and percentage of tumour necrosis after induction CT. Gorlick., *et al.* [17] points out that the current literature concludes that there is no correlation between P-gp status and percentage of OS tumor necrosis after induction chemotherapy. Pakos., *et al.* [18] did a meta-analysis of 14 studies ($n = 631$ patients) that evaluated the correlation between P-gp and histologic response to chemotherapy and clinical disease progression and showed that P-gp was not associated with the histologic response of patients with OS to combination CT regimens. Conversely, P-gp positivity, as determined by IHC, was a strong correlate of more rapid disease progression. In the present study the overall survival was also studied in P-gp positive cases. In our study the cumulative survival was low in cases with moderate intensity and was 12.5% and those with weak intensity were 60%. Those with strong intensity did not survive more than 1 year. This survival difference was statistically significant ($p = 0.02$). This was consistent with previous studies. In a study by Serra., *et al.* [19] P-Gp positivity was found in 47 of 149 cases (32%) and was significantly associated with a higher incidence of relapse and a worse outcome. Baldini., *et al.* [20] assessed P-glycoprotein status in relation to the length of event free survival in 149 patients with primary, non-metastatic, high-grade OS. P-glycoprotein positivity was found in 47 of 149 cases

(32%). It was shown that a wide spread pattern of P-Gp expression in tumor cells at diagnosis was significantly associated with a higher rate of systemic relapse ($p < 0.001$). In another study Baldini., *et al.* [21] concluded that the presence of increased levels of P-glycoprotein in the OS was significantly associated with a decreased probability of remaining event-free after diagnosis ($P = 0.002$). On analysing the level of expression of P-glycoprotein and the extent of tumor necrosis after preoperative CT; there was over expression of P-glycoprotein found in tumours from 6 of 25 patients (24 percent) with a poor response (90 percent tumor necrosis) and from 22 of 67 patients (33 percent) with a good response (90 percent tumor necrosis), ($P = 0.46$). In a multivariate analysis, P-glycoprotein status ($P = 0.001$) and the extent of tumor necrosis after preoperative chemotherapy ($P = 0.04$) were independent predictors of clinical outcome. Wunder., *et al.* [22] analysed tumours from 123 patients for MDR1 mRNA expression and the association of the level of MDR1 expression with the risk of systemic recurrence was examined using survival analyses with traditional and histologic markers as prognostic factors demonstrated no correlation between MDR1 gene expression and prognosis (R.R, 1.15; $P = .44$). Schwartz., *et al.* [23] in a prospective analysis on specimens derived from children and young adults with osteosarcoma enrolled onto a National Intergroup trial (INT0133) did not find that immunohistochemical analysis of P-gp expression predicted outcome for patients. A study by Ferrari., *et al.* [24] showed that the patients with high grade OS exhibited increased cellular expression of P-gp, ErbB-2, and Bcl-2 in pulmonary metastases compared with primary tumour. In the present study Her-2 expression in tumour cells of cases with osteosarcoma was done by IHC. In this study we studied 36 cases for the expression of Her2/neu in formalin - fixed paraffin embedded tissue sections. All cases were negative for Her-2. Few cases were submitted for Her-2 amplification which showed no signals. In a study by Maitra [25] they investigated the status of both the HER-2/*neu* oncogene and its protein product p185c-erbB2 in a cohort of 21 paediatric osteosarcomas by the simultaneous use of FISH and IHC. None of the 21 osteosarcomas had evidence of HER-2/*neu* gene amplification by FISH. p185c-erbB2 IHC was also negative in all cases. These results showed that HER-2/*neu* gene amplification appeared to be an uncommon event in paediatric OS.

Overall survival was analysed and the median survival time was 19 months. The range was from minimum of 2 months and maximum 104 months. Median Follow Up time was 13 months. The 5-year survival of the paediatric patients with OS in our study was 30.73%. The 3-year survival was 70.59%.With the modern treatment approximately 60 - 70% of newly diagnosed, resectable osteosarcoma patients can expect to be disease free 3 years from diagnosis. The outcome for patients with initially metastatic disease and for those who develop recurrent disease remains much worse with reported 2-year survival of 10 - 30%. French Bone Study Group [26] showed that the 3-year overall survival was 61%. Study by Glasser., *et al*. [27] showed a 5-year survival of 77% but patients with metastatic disease at the time of presentation were excluded.

Presence of metastasis at the time of presentation was another factor which was studied. In our study 22 cases showed metastasis ($n = 22$). It was seen that metastasis at presentation had significant correlation with survival. Lung was the most common site of metastasis followed by skeletal metastasis. This was consistent with previous studies which showed that presence of metastasis is an important prognostic factor. According to Marina N., *et al*. [28] the presence of metastatic disease most commonly to the lungs was the most important prognostic factor.

Conclusion

In this study, location of tumor in long bones, presence of metastasis, intensity of positivity of P-glycoprotein is the factors which had prognostic significance. Her-2/neu was negative in all cases of osteosarcoma. P-gp positivity had no correlation with histologic response to chemotherapy. Histologic response to NACT had no correlation with survival. Further studies with more sample size and regular follow up would be needed to reach a consensus on this subject.

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