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The effect of resistance-related proteins on the prognosis and survival of patients with osteosarcoma: an immunohistochemical analysis

Osteosarkomlu hastalarda dirençle ilişkili proteinlerin prognoz ve sağkalım üzerine etkisi: İmmünhistokimyasal analiz

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Amaç: Kemoterapi rejimlerindeki gelişmelere rağmen osteosarkom sağkalımında çok az ilerleme olmuştur. Bu çalışmada, osteosarkomlu hastalarda prognoz ile ilişkili bazı proteinlerin sağkalım ile ilişkisi değerlendirildi.

Çalışma planı: Tedavisi ve izlemi hastanemizde yapılan 45 hastanın (24 erkek, 21 kadın) verileri geriye dönük olarak incelendi. Osteosarkom nedeniyle, neoadjuvan kemoterapi sonrasında, 41 hastaya ekstremite koruyucu cerrahi, dört hastaya amputasyon yapılmıştı. En sık tutulum 23 hasta (%51.1) ile femur alt uç, 10 hasta (%22.2) ile tibia üst uçta görüldü. Üç hastada başvuru anında metastaz vardı. Cerrahi rezeksiyon örnekleri patoloji arşivinden çıkartılarak, p-glikoprotein p170, p53, ısı şok proteini 27 (HSP27), HSP90 ve nm23 proteinlerinin ekspresyonu immünohistokimyasal yöntemlerle incelendi. Bu proteinlerin prognoz ve sağkalım üzerindeki etkileri Kaplan-Meier yöntemi ile değerlendirildi. Hastaların ortalama takip süresi 49.7 ay (dağılım 6-185 ay) idi.

Sonuçlar: Başvuru anında metastaz saptanan üç hasta beş yıl içinde akciğer metastazından yaşamını yitirdi. Yirmi dokuz hastada metastaz gelişti. Beş ve 10 yıllık genel sağkalım oranları sırasıyla %60 ve %43 bulundu. Hastalıksız sağkalım oranı ise beş yıl için %41, 10 yıl için %24 idi. Metastaz gelişen hastalarda beş yıllık sağkalım oranı %29 idi. Klinik faktörler içinde, sağkalımı anlamlı etkileyen sadece başvuru anında metastaz varlığı idi (p=0.044). p53 proteininin pozitif ve negatif ekspresyonları arasında beş yıl ve on yıllık sağkalım oranları açısından anlamlı fark görülürken (p=0.04), incelenen diğer proteinler sağkalımla ilişkili bulunmadı.

Çıkarımlar: p53 ekspresyonu ile sağkalım arasındaki ilişki, p53'ün osteosarkomda prognostik gösterge olarak kullanılabileceğini düşündürmektedir.

Anahtar sözcükler: İlaç direnci, neoplazi; immünhistokimya; neoplazi proteinleri; osteosarkom; prognoz; sağkalım analizi; tümör belirteci, biyolojik; tümör supresyon proteini p53.

Objectives: Despite the developments in chemotherapy protocols, improvement in the survival rates of osteosarcoma has been limited. We evaluated the effect of certain prognosis-related proteins on survival of patients with osteosarcoma.

Methods: Data from 45 patients (24 males, 21 females) who were treated and followed-up for osteosarcoma were reviewed. Following neoadjuvant chemotherapy, 41 patients underwent extremity saving surgery, and four patients underwent amputation. The most frequent localization was the lower end of the femur (n=23, 51.1%), followed by the upper end of the tibia (n=10, 22.2%). Three patients had metastasis on admission. Surgical resection samples were retrieved from the pathology archive and analyzed immunohistochemically for the expression of p-glycoprotein p170, p53, heat-shock protein 27 (HSP27), HSP90, and nm23. The effect of these proteins on prognosis and survival was assessed with survival analysis using the Kaplan-Meier method. The mean follow-up was 49.7 months (range 6 to 185 months).

Results: Three patients with metastasis on admission died within five years due to pulmonary metastasis. New metastases developed in 29 patients. Total 5-year and 10-year survival rates were 60% and 43%, respectively. The corresponding disease-free survival rates were 41% and 24%. Five-year survival was 29% in patients who developed metastasis. Among clinical factors, survival was influenced only by the presence of metastasis on admission (p=0.044). Five-year and 10-year survival rates were significantly different between patients with and without p53 positivity (p=0.04), while the other proteins were not significantly associated with survival.

Conclusion: Our data suggest that p53 may be used as a prognostic marker in osteosarcoma due to its significant association with survival.

Key words: Drug resistance, neoplasm; immunohistochemistry; neoplasm proteins; osteosarcoma; prognosis; survival analysis; tumor markers, biological; tumor suppressor protein p53.

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Osteosarcoma is the most common primary malignant bone tumor in pediatric and adult patients. In the last years, total survival rates have risen to 60-70% at many centers after the introduction of novel neoadjuvant and adjuvant chemotherapy protocols. Nevertheless, a large group of patients die due to systemic disease. Further improvement in survival rates have not been achieved because of the insufficient chemotherapy response rate of a certain group of patients. The reason of inadequate response to chemotherapy in some patients is thought to be related to the emerging drug resistence for chemotheraupetics in the last years.[1]

In this study, the association between the survival rates of osteosarcoma patients who were treated in our hospital and certain factors like age, sex, anatomical location, histological type, the presence of pathologic fracture, the presence of metastasis at presentation, and certain prognosis-related proteins which were previously shown to be significant in various cancer types was retrospectively analysed.

Patients and methods

Pathology reports of 185 patients who were diagnosed to have osteosarcoma between 1990 and 2006 were reviewed. Patients whose primary biopsies were performed and were followed up at the same hospital were included in this study, while patients whose materials were not technically adequate for application of antibodies were excluded. Fifty patients wereconsequently included in the study . Twent two patients were in pediatric age group (age ≤16), and 28 patients were older than 16 years of age. Preoperatively, patients of peditric age group had received cisplatin, ifosfamide and epirubicin treatment; and older patients had received adriablastin/cisplatin; adriablastin/ifosfamide or methotrexate treatment. Following neoadjuvant therapy, limb salvage surgery was applied to 41 patients and 4 patients had amputation. After both groups had undergone surgical resection they completed their chemotherapy protocols postoperatively. The preoperative biopsy materials of five patients were inaccessible, so they were excluded from the study due to insufficient data. Consequently, hematoxylin & eosin stained slides of 45 patients (24 males and 21 females; mean age 20; distributed between 8-64 years) were retrieved from the archive and re-examined. In addition, paraffin blocks of the studied slides, which were previously formalin-fixed and decalcified with nitric acid, were recovered.

The following proteins were studied with immunohistochemical methods on sections of the retrieved parafin blocks: (i) p53 protein, which was previously shown to have a negative prognostic effect on various cancer types;[2-6] (ii) heat shock proteins HSP27 and HSP90, which were shown to have different prognostic effect for different cancer types while positive expression is usually associated with bad prognosis for human osteosarcoma;[7,8] (iii) p glycoprotein p170, which is a plasma membrane protein produced by a multidrug resistance (MDR) gene; [9,10] (iv) nm23 protein, which was found to be produced by a metastasis suppressor gene.[11-14]

For the immunohistochemical detection of p-glycoprotein antigen, p170 MDR Ab-2 (Clone F4) mouse monoclonal antibody (Neomarkers,cat.no:MS-660-P1, Fremont, CA, USA); for the immunohistochemical evaluation of p53 protein, p53 Ab-5(Clone DO-7) mouse monoclonal antibody (Neomarkers,cat no : MS-186-PO, Fremont, CA,USA); for heat shock protein 27, HSP-27 Ab-1(Clone G3.1) mouse monoclonal antibody (Neomarkers,cat no:MS 101-PO, Fremont, CA, USA); for heat shock protein 90_, HSP86 Ab-1 rabbit policlonal antibody (Neomarkers,cat no :RB-119-PO, Fremont , CA, USA); and for nm23 protein, NCL-nm23 mouse monoclonal antibody (clone 37.6, Novocastra, Newcastle, UK) were used.

The following materials were used commonly for all antibodies: secondary antibody (Lab Vision Corp.cat.no:TP-125-HL, Fremont, CA, USA), nonspesific block (Lab Vision Corp.cat.no:TA-125-UB, Fremont, CA, USA), streptavidine peroxidase (Lab Vision Corp.cat.no:TS-125-HR,Fremont, CA, USA), AEC chromogen (Lab Vision Corp.cat.no: TA-004-HAC) and AEC substrate (Lab Vision Corp.cat.no: TA-060-HA).

Immunohistochemical method

In order to apply p-glycoprotein, p53, nm23, HSP 27 and HSP86 immunohistochemical antibodies on selected paraffin blocks, five 3-5 mm sections were taken from each block on poly-L-lysine coated slides. These sections were left at a 56°C stove for approximately 12 hours to dry and stick to the slides. Later,the slides were bathed with xylene 6 times for deparaffinisation and were left for 30 minutes. The

Table 1. The distribution of tumor localizations

Localization	Number	Percentage
Distal femur	23	51.1
Proximal tibia	10	22.2
Humerus	5	11.1
Tibia diaphysis	5	11.1
Femur diaphysis	1	2.2
Other	1	11.1

slides were then presented to 100% ethyl alcohol, followed by 96% ethyl alcohol for 6 turns and were left for 15 minutes each time, and finally were hydrated with distilled water.

2.1 grams of citric acid was dissolved in 1 L. distilled water for antigen retrieval procedure for slides on which p53, nm23, HSP27 and HSP90 would be applied. The pH of the solution was set to 6 with 2 normals of NaOH. The prepared citrate buffer was heated until boiling in a pressure cooker, while the lid was closed but the pressure valve was left open. The previously hydrated slides were then placed into the boiling citrate buffer. After making sure that the sections were completely covered with the citrate buffer, the lid was closed. The pressure valve of the pressure cooker was left open. Heating process continued until steam came out from the pressure valve instead of air. When steam came out, the pressure valve was closed and heating was continued for 3 minutes. After 3 minutes, the cooker was taken away from the heater and was left to cooling at room temperature for 20 minutes. After the cooker was cooled down to a safe level, pressure valve and lid was opened and cooling process continued. When the 20 minutes expired, the sections were taken in distilled water. The slides prepared by this process were then examined under light microscope. Expression levels of studied proteins were determined according to the staining levels of proteins at the slides.

In order to assess the influence of these proteins on prognosis and survival, survival analyses were carried out with Kaplan-Meier method, using SPSS 13.0 program for statistical analysis. In addition, some prognostic factors, which are proposed to have an effect on survival, were assessed with univariate and multivariate analyses. The mean follow-up period was 49.7 months (6-185 months).

Results

Tumor localizations are shown in Table 1. The most common localizations were distal femur (23 patients, 51.1%) and proximal tibia (10 patients, 22.2%).

Three patients had metastasis at presentation. Those 3 patients died of lung metastasis in 5 years. 27 patients suffered from lung metastasis and 2 patients had metastasis other than lung.

Total survival rate was 60% in 5 years and 43% in 10 years. Disease free survival rate was 41% in 5 years and 24% in 10 years (Figure 1). The significance of some prognostic factors on survival such as age, gender, anatomic localization, histological type, the presence of pathological fracture, and the presence of metastasis at presention was assessed with Kaplan-Meier statistical test. A weak negative correlation was found between age and survival rate but there was no statistical significance (p>0.05). Likewise tumor lo-

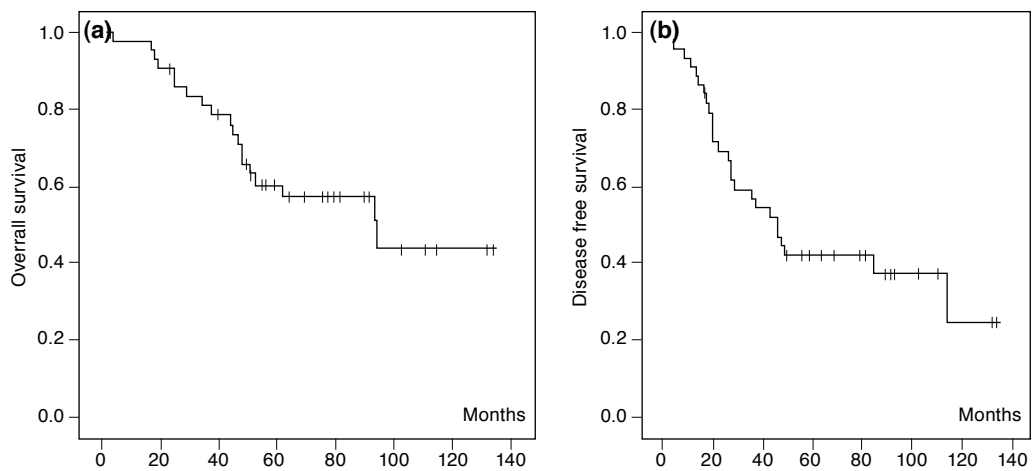

Figure 1. (a) Total survival rate for all patients. **(b)** Disease-free survival curve

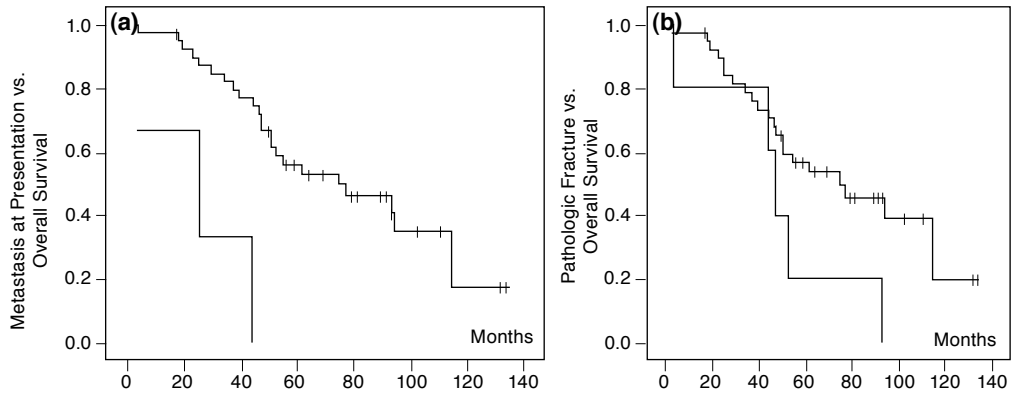

Figure 2. (a) Graph shows the significant relationship between the presence of metastasis at presentation and survival. All patients who had presented with metastasis were dead by 40. month. **(b)** Graph showing the relationship between the presence of pathological fracture and survival.

calization, presence of pathologic fracture, and chief complaint at presentation had no statistically significant effect on prognosis.

Five-year survival rate of metastatic patients was 29±8. The rate of necrosis was graded according to Huvos criteria, and was divided into two groups (0-94% and 94-100%) for statistical analysis. First group included 31 patients and second group had 13 patients. Necrosis rate of one patient was inaccessible. At the 0-94% necrosis group, 5 year survival rate was 43±9% and 10 year survival rate was 14±8%; and at the 94-100% group 5 year survival rate was 69±12%. Among all studied factors, only the presence of metastasis at presentation was shown to have a statistically significant effect on survival (p=0.044). For the patients who had presented with pathological fracture, there was a tendency for poorer prognosis but no statistical significance was found (Figure 2).

Protein expression levels and survival analysis

The expression levels of p53, p170, nm23, HSP27 and HSP90 proteins were recorded as (-): no staining; (+): <1-10% staining; (++): 10-70% staining; (+++) 70-90% staining; and (++++): 90-100% staining. For statistical analysis, staining less than 10% was considered as a negative result and staining more than 10% was considered as a positive result; and these two groups were used for analysis (Table 2).

Table 2. The expression levels of studied proteins

	Positive	Negative	*p*
p53	23	22	**0.04**
p170	43	2	–
nm23	7	38	0.59
HSP27	25	20	0.95
HSP90	3	42	–

5 year total survival rate of 23 patients who were positive for p53 was 48±10% and 10 year survival rate was 31±12%. For the 22 pateints who were negative for p53, 5 year survival rate was 71±10%, and 10 year survival rate was 53±17%. There was a statistically significant difference between groups with positive or no expression of p53 protein (p=0.04). Prognosis was worse for patients who were p53 positive (Figure 3).

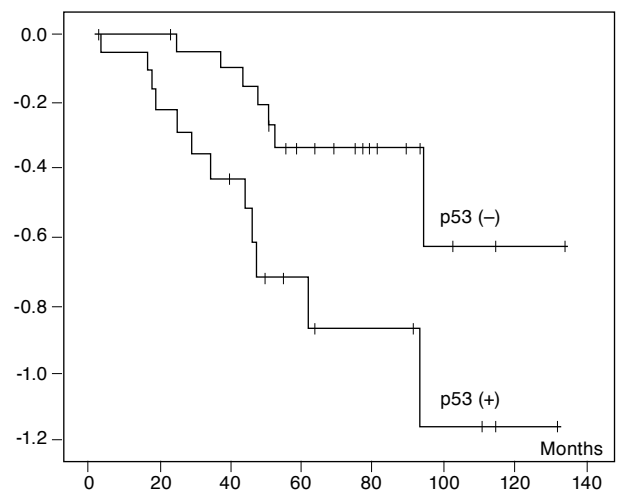

Figure 3. Graph shows the relationship between p53 expression and survival. The lower line represents the group which shows p53 expression and the upper line represents the group with negative p53 expression. As time passes, survival rate falls more rapidly in the group with positive p53 expression while it remains higher in the second group.

Five year survival rate of 7 patients who were nm23 negative was 62±21; for the 38 patients who were nm23 positive, 5 year survival rate was 60±8 and 10 year survival rate was 38±12%. There was no significant difference between two groups (p=0.59).

For the 25 patients who were HSP27 negative, 5 year survival rate was 62±10, and 10 year survival rate was 41±12%; for the 20 patients who were HSP27 positive, 5 year survival rate was 57±11% but 10 year survival rate was not determined. There was no statistically significant difference (p=0.95). Statistical analysis for HSP90 and p170 proteins was not possible because of their uneven distribution of expression between groups.

Discussion

The heterogenous study group in this present study had a 10 year total survival rate of 60% and a disease free survival rate of 44%. Despite the fact that osteosarcoma treatment is standardized throughout the world, 5 year total survival rate varies from 50% to 70% in various major centers.[15-20] The most comprehensive study belongs to the German-Swiss-Austrian Cooperative Study Group, which is a series of 1702 cases, and in this study the 10 year total survival rate was found to be 59.8%.[16] The Rizzoli Institute reported 10 year survival rate as 70% and disease free survival rate as 59%, excluding patients who had presented with metastasis at the beginning. [15] The survival rate determined in our study conforms with the aforementioned studies.

Considering factors that have an effect on prognosis, as also previously shown in various studies, the presence of metastasis at first presentation had a significant effect on prognosis in this study. A significant association between pathologic fracture and poor prognosis has been shown in some previous studies.[21] In this study, however, the presence of pathologic fracture indicated no significant effect on prognosis despite a tendency for poor prognosis was present.

Mutations in p53 tumor suppressor gene have been reported to have a role in the pathogenesis and cell proliferation in osteosarcoma. In various studies, p53 expression is shown to be associated with poor prognosis in various types of cancer such as breast, gastric, colon, rectum, bladder, and non small cell lung cancer. [2-6] However, no such correlation was detected for osteosarcoma in most studies, and though an association was shown in a study, it wasn't clear-cut because of low sample size and lack of statistical reliability.[22] In a study conducted by Wadayama et al, p53 expression was shown in 19 out of 67 (28.4%) osteosarcoma cases and no significant difference in expression was found between primary and metastatic tumors.[16] While a clinical correlation was shown in many studies with a p53 positivity rate of only 15-25% [5]; the importance of the present study is the strong statistical relationship between p53 positivity and survival which indicates that p53 may be used as a prognostic marker in osteosarcoma.

Despite the developments in diagnosis and treatment, the most common cause of death in cancer patients is metastasis which is resistant to conventional treatment. There is evidence that decreased nm23 expression may be associated with metastasis in cancer types such as breast, ovarian, gastric carcinoma, hepatocellular carcinoma and melanoma.[11-14] In immunohistochemical and molecular studies on osteosarcoma, nm23 is shown to have a role in metastatic progression rather than metastasis suppression.[23] In these studies, expression rate was higher at metastasis sites when compared with primary tumor site. The studies on osteosarcoma have found no statistically significant relationship between nm23 expression and early metastasis; nevertheless, cases with positive nm23 expression have been shown to have a tendency for early metastasis.[11] Likewise, the present study failed to show a significant relationship between nm23 expression and survival; yet, nm23 positive cases had a higher rate of metastasis and death.

Heat shock proteins are a group of proteins whose expression is increased during cellular stresses such as heat and drug exposure. In the literature, HSP27 has been shown to be associated with poor prognosis in osteosarcoma and ovarian carcinoma patients considering survival rate.[13,14,24,25] In a limited number of studies on HSP and osteosarcoma, HSP90 expression has been found to have a good prognostic value.[5,13] However, another study has shown that there is no association between HSP90 expression and prognosis in osteosarcoma.[8] In the present study, heat shock proteins failed to demonstrate any significant influence on the survival rate.

P170 (p glycoprotein) was another protein that was examined in the present study. There are some studies in the literature which shows that positive expression of p170 leads to poor prognosis.[26] A study by Serra et al showed that cell lines showing overexpression of p-glycoprotein need more aggressive chemotherapy because of multidrug resistance. [9] In our study, no statistical evaluation was possible for p170 because of uneven distribution.

We acknowledge the weaknesses of the study. Some statistical incompetence is mentioned above. In addition, the lack of a control group in the study and the lack of two different groups who were resistant and not resistant to chemotherapy are weaknesses of the study.

Conclusıon

In this study, P53 positivity is shown to indicate poor prognosis in osteosarcoma by immunohistochemical studies. It is not shown that heat shock proteins (HSP27 and HSP90), nm23 and p170 proteins, which have been previously shown to have prognostic effects for various cancer types, have a statistically significant effect on the prognosis of osteosarcoma. Different results may be found if the prognostic effects of those proteins are studied in studies with wider sample size.

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