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ORIGINAL

Investigation of the prognostic significance of c-kit and c-erb-b2 expression in osteosarcoma, ewing sarcoma, and rhabdomyosarcoma and comparison with clinical parameters

Investigación de la importancia pronóstica de la expresión de c-kit y c-erb-b2 en osteosarcoma, sarcoma de ewing y rabdomiosarcoma y comparación con parámetros clínicos

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Abstract

Objective: In the present study, the prognostic and treatment-guiding roles of the c-erb-B2 oncoprotein and tyrosine kinase receptor c-kit in osteosarcoma (OS), Ewing sarcoma (ES), and rhabdomyosarcoma (RMS) were evaluated.
Materials and methods: With 25 cases from each group, c-kit and c-erb-B2 expression were investigated immunohistochemically in a total of 75 cases.
Results: Staining for c-kit was found to be positive in 15 (60%) of the RMS, 11 of the OS (44%), and 11 of the ES cases (44%). C-erbB-2 staining was positive in 7 RMS cases (8%) and negative in all cases of OS and ES.The SISH method was applied to six c-erb-B2 positive RMS cases, and amplification of the HER2 gene (encoding c-erb-B2) was detected in one case. It was determined that c-kit expression in OS was significantly associated with survival ($p<0.05$) and could be considered as a poor prognostic parameter. In contrast,c-kit expression had no prognostic significance in ES and RMS. In the RMS group, there was no relationship between c-erb-B2 positivity and prognosis.
Conclusion: It was concluded that c-kit expression can be used as a poor prognostic parameter for OS and therapy targeting c-kit may be an alternative treatment option.

Keywords: Osteosarcoma, Ewing Sarcoma, Rhabdomyosarcoma, C-Kit, c-erb-B2.

Resumen

Objetivo: En el presente estudio, se evaluaron las funciones de guía de tratamiento y pronóstico de la oncoproteína c-erb-B2 y el receptor de tirosina quinasa c-kit en osteosarcoma (OS), sarcoma de Ewing (ES) y rabdomiosarcoma (RMS).
Materiales y métodos: Con 25 casos de cada grupo, se investigó inmunohistoquímicamente la expresión de c-kit y c-erb-B2 en un total de 75 casos.
Resultados: Se encontró que tinción para c-kit fue positiva en 15(60%) de RMS, 11 de casos de OS (44%) y 11 de los casos de ES (44%). La tinción de C-erbB-2 fue positiva en 7 casos de RMS (8%) y negativa en todos los casos de OS y ES. Se aplicó el método SISH a seis casos de RMS positivos para c-erb-B2, la amplificación del gen HER2 (que codifica c-erb-B2) se detectó en caso. Se determinó que expresión de c-kit en OS se asoció significativamente con supervivencia ($p<0,05$), podría considerarse como un parámetro de mal pronóstico.
Conclusión: se concluyó que la expresión de c-kit se puede utilizar como un parámetro de mal pronóstico para OS y que la terapia dirigida a c-kit puede ser una opción de tratamiento alternativa.

Palabras clave: Osteosarcoma, sarcoma de Ewing, rabdomiosarcoma, C-Kit, c-erb-B2.

Introduction

Bone and soft tissue sarcomas constitute a large group of malignancies diagnosedin childhood and young adulthood. Osteosarcoma (OS) is the most commonly observed primary malignant bone tumor in this group; while it accounts for approximately 20% of primary malignant bone tumors, it comprises 56% of childhood malignant bone tumors[1-3]. The second-most common malignant bone tumor after OS is Ewing sarcoma (ES)[4,5]. Rhabdomyosarcoma (RMS), on the other hand, accounts for 19% of soft tissue sarcomas diagnosed in adolescents and young adults[6].

Despite advances in treatment methods forbone and soft tissue sarcomas, the desired improvements in survival rates have not been achieved, especially in metastatic and recurrent cases. In recent years, biological inhibitors targeting proteinsthat play a role in oncogenesis have been introduced into treatment regimens and positive results have been obtained[7,8].

C-kit (CD117) is a proto-oncogene encoding the transmembrane cell surface receptor kit. It is a member of the same subclass of genes as platelet-derived growth factor and colony-stimulating growth factor. Functional mutations inc-kit have been associated with the pathogenesis of a number of malignancies expressing c-kit, including gastrointestinal stromal tumors, mast cell disease, and acute leukemia[9].

The c-erb-B2 receptor (encoded by the *HER2* gene) belongs to the epidermal growth factor receptor family and plays a crucial role in the activation of the subcellular signal transduction pathways that control the growth and differentiation of epithelial cells. Monoclonal antibody therapy directed against the extracellular region of c-erb-B2 has long been used to treat breast and stomach carcinomas[10-13].

Our research aims to investigate c-kit and c-erb-B2 protein expression in OS, ES, and RMS cases; to determine their relationships with clinical parameters, and to determine their effect on prognosis.

Materials and Methods

A total of 75 patients diagnosed with OS, ES, or RMS (25 from each group)in the authors' institution were randomly selected. The patients' clinical and macroscopic information, such as age, gender, resection type, and survival time, were obtained by reviewing the archived reports and reports from oncology clinic follow-ups. The archived slides were re-evaluated histopathologically, and histological classification was performed for the OS and RMS cases.

Immunohistochemistry

A total of four new 4 µm sections were prepared from the selected paraffin blocks, including one for H&E examination, two for immunohistochemical (IHC) examinations, and one as a backup. In this study, c-erb-B2 (monoclonal, mouse anti-human, Code: NCL-L-CBE-356, Clone: 10A7, 1:100, 60 min, Lot no: L 135613, Novocastra, Newcastle, UK)and c-kit/CD117 (Polyclonal, rabbit anti-human, Code: RB-9038-P1, 1:100, 60 min, Lot no: 9038P412F, NeoMarkers, Fremont, CA, USA) antibodies were used to evaluate the expression of their respective targets using the standard avidin-biotin-peroxidase complex technique. Gastrointestinal stromal tumors and breast carcinoma were used as positive controls for c-kit and c-erb-B2, respectively.

Immunohistochemical evaluation

In the IHC evaluation using the c-kit antibody, the percentage of positive cells was categorized into three groups as follows: staining in 0%–10% of tumor cells, in 10%–50% of tumor cells, or in >50% of tumor cells. In addition, the staining intensity was categorized as weak (1+), moderate (2+), or strong (3+). Results were accepted to be negative if staining was observed in 0%–10% of tumor cells and the staining intensity was (1+), positive if 0%–10% of tumor cells were stained and the staining intensity was (2+) or (3+), and positive if staining was observed in 10%–50% or>50% of tumor cells and the staining intensity was (1+), (2+), or (3+).

In the IHC evaluation using the c-erb-B2 antibody, the percentage of stained cells was categorized into two groups: staining of 0%–10% of tumor cells or >10% of tumor cells. In addition, the staining intensity was classified as weak, moderate, or strong, with staining visible with the x2 or x4 microscope objective considered strong, with the x10 or x20 objective considered moderate, and with the x40 objective considered weak. The localization of staining in the cells was indicated as membranous or cytoplasmic-membranous. The results were considered negative if <10% of tumor cells had staining (regardless of staining intensity) and negative (1+) if more than 10% of tumor cells had staining, but the staining was weak. The results were considered positive (2+) if >10% of tumor cells were stained and the staining intensity was moderate and positive (3+) if >10% of tumor cells had strong staining[12,14].

Silver in situ hybridization

For silver in situ hybridization (SISH), the inform HER2 DNA probe kit (VENTANA) was used with the VENTANA Benchmark LT instrument. Microscopic examination was performed on the slides treated with c-erb-B2 and chromosome 17 DNA probes.

Evaluation of the silver in situ hybridizationmethod

Nuclear signals of a total of 40 cells were counted in

at least two fields that met the scoring criteria and the average number of signals per nucleus was determined. If the average number of signals for c-erb-B2 (*HER2*) divided by the average number of signals for chromosome 17 was >2.2, it was considered significant.

Statistical evaluation

For cross-sectional variables, Chi-square, Fisher's exact, Kaplan–Meier, andlog-rank tests were performed for statistical evaluation. $p<0.05$ was considered significant. Statistical analyseswere performedusing SPSS 8.0 software.

Results

Clinicopathological results

The peak incidence of disease in the OS group, which consisted of 25 cases, was in the second decade. The distribution of histological types according toage and gender is presented in **table I**. The most common location was the extremities (15 cases; 60%). Of those, nine (36%) were in the femur and six (24%) were in the tibia. Other locations were the mandible in three (12%) and the pelvis in two (8%) of the cases. It was determined that the patients had an average follow-up period of 25.4 months. Thirteen of the patients had died due to disease-related causes, one patient had local recurrence, and one patient had metastasis. The remaining nine patients are alive and healthy, with no symptoms of disease.

The patients were divided into two age groups (under 18 years of age and 18 years of age and older), and the correlation with gender, localization, histological type, and survival was investigated. There was no statistically significant difference between the two age groups. In addition, no difference in correlation was observed between the age groups with regard to histological type, tumor location, and survival. Necrosis rates, which were divided into two groups (below 90% and above 90%), were found not to be correlated with histological type orsurvival.

The age and gender distributions of the 25 ES cases are shown in **table II**. The peak incidence was determined to be the second decade. The most common site of involvement in ES cases wasthe lower extremities (52%), while the other locations were the trunk (20%), upper extremity (12%), head-neck (12%), and 4% lymph nodes (4%).

Clinical follow-up data were obtained for15 of the 25 cases. The mean follow-up period was 23.2 months. Eight of the patientshave died due to disease-related causes, and metastasis was detected in four of the patients. The remaining three patients are alive and healthy, with no symptoms of disease.

In the statistical analysis, it was determined that gender was not correlated with survival. There was no significant difference ingender, tumor location,orsurvival according to age group (cases were divided into two groups, <18 years old and ≥18 years).

The association of age and gender with histological type in RMS cases is shown in **table III**. The distribution of the tumor locations was head-neck (36%), trunk (24%), urogenital region (16%), upper extremity (12%), lower extremity (8%), and lymph nodes (4%).

In the 14 (out of 25) cases whose clinical follow-up records were accessed, the follow-up period ranged between14 and 51 months, with an average of 27.6 months. Two patients have died due to disease-related causes, one patient had local recurrence, and four patients had metastasis. The remaining seven patients are alive and healthy, with no symptoms of disease.

There was no statistically significant difference between male and female patients with RMS in terms of mean age and survival. Gender, localization, histological type, and survival rates were found to be similar between the two age groups (under 18 years and over 18 years old). Histological types, localization, and survival were found not to be correlated with age group.

Immunohistochemistry results

C-kit

Cytoplasmic positivity was detected in 11 (44%) of the OS cases (**Figure 1A**). In some cases, cytoplasmic staining was accompanied by a weak membranous staining. Of the positive cases, two (8%) had diffuse-strong, six (24%) had diffuse-moderate, and the remaining three (12%) had focal, weak-moderate staining. Weak staining was observed below 10% in 3of the 14 cases evaluated as negative. Cytoplasmic positivity was observed in osteoblastic and osteoclastic cells in one of the negative cases. The association between c-kit expression leveland overall survival is shown in **Graphic 1**. A statistically significant correlation was found between c-kit positivity andoverall survival ($p<0.05$) (**Table IV**, **Graphic 1**). In addition,a result at the limit of statistical significance was obtainedbetween the intensity of c-kit expression and survival ($p>0.05$). However, there was no difference between the c-kit expression groups in terms ofclinicopathological characteristics, including age, gender, localization, histological type, and response to neoadjuvant chemotherapy.

Similar to OS,c-kit expression was positive in 11 (44%) patients with ES (**Figure 1B**). No staining was observed in 14 cases (56%). In eight of these cases, weak staining was detected in below 10% of tumor cells, which was evaluated as negative. Staining was diffuse-strong in

two positive cases (8%), diffuse and weak-moderate in four cases (16%), and focal and weak-moderate in the remaining five cases (20%). Mean survival was 13 months in cases with c-kit expression and 41 months in c-kit–negative cases; however, no significant correlation was found between c-kit immunoreactivity, overall survival, age, gender, ortumor location.

Positive cytoplasmic staining was found in 15 (60%) of the 25 RMS cases (**Figure 1C**). Diffuse and strong staining was detected in 3 cases (12%), diffuse and weak-moderate staining was observed in 6 cases (24%), and focal and weak-moderate staining was observed in the remaining 6 positive cases (24%). In 4 of the 10 (40%) negative cases, weak staining in less than 10% of tumor cells was observed.

The association of c-kit expression with overall survival and histological type is presented in **table V**. The mean survival was determined to be35 months in cases with c-kit expression and 43 months in cases with no c-kit expression. There was no statistically significant correlation between c-kit immunoreactivity and survival, age, gender, tumor location, or histological type (p>0.05).

C-erb-B2

No staining was detected with thec-erbB-2 antibody in OS and ES cases, whereas positive staining was observed in 7 (28%) of the RMS cases (**Figure 1D**). Of these cases, 4 (16%) were '2+' and 3 (12%) were '3+'. A membranous-cytoplasmic staining pattern was observed in all postive cases (28%). It was noted that rhabdomyoblastic differentiation was distinct in positive cases. C-erbB-2 expression was not found to be correlated with overall survival (p>0.05).

Silver in situ hybridization

The SISH test was performed in6 of the 7 RMS cases that stained positive with c-erb-B2(1 case could not be studied due to the absence of tissue in the paraffin block) to detect *HER2* gene amplification. HER2 gene amplification was detected in one patient (**Figure 1E**).

Table I: Gender and age distribution of osteosarcoma cases according to histological types.

Histological Type	Number of Cases (%)					
	Total	Female	Male	Age Distribution	Mean±SS	Median
Osteoblastic	11	8 (73)	3 (27)	14-29	19.82±5.07	17.00
Chondroblastic	11	4 (37)	7 (63)	11-52	23.36±11.89	18.00
Fibroblastic	2	2 (100)	-	21-34	27.5±9.19	27.5
Telangiectatic	1	-	1 (100)	39	39.00±0.00	39
Total	25	14 (56)	11 (44)	11-52	22.76±9.49	19

Table II: Gender and age distribution in Ewing sarcoma cases.

Gender	Number of cases	Average Age	Standard Deviation	Age Distribution
Male	16	27.50	19.58	10-80
Female	9	20.56	11.62	3-35
Total	25	25.00	17.21	3-80

Table III: Gender and age distribution of rhabdomyosarcoma cases according to histological types.

	Number of Cases (%)					
	Total	Female	Male	Age Distribution	Mean	Standard Deviation
Alveolar	12	6 (50)	6 (50)	1-35	14.8	11.76
Embryonal	9	6 (67)	3 (33)	2-74	16.9	23.06
Spindle Cell	2	1 (50)	1 (50)	14-17	15.5	5.19
Pleomorphic	1	-	1 (100)	6	6	0.00
Botryoid	1	1 (100)	-	25	25	0.00
Total	25 (100)	14 (56)	11 (44)	1-74	15.7 (1-74)	15.79

Table IV: Distribution of C-kit immunoreactivity according to histological type and survival in osteosarcoma cases.

		Number of Cases (%)	C-kit Positive (%)	C-kit Negative (%)
Histological Type	Osteoblastic	11 (44)	4 (37)	7 (50)
	Chondroblastic	11 (44)	5 (45)	6 (43)
	Fibroblastic	2 (8)	2 (18)	-
	Telangiectatic	1 (4)	-	1 (7)
	Total	**25 (100)**	**11 (100)**	**14 (100)**
Survival	Live	11 (46)	3 (30)	8 (57)
	Dead	13 (54)	7 (70)	6 (43)
	Total	**24 (100)**	**10 (100)**	**14 (100)**

Table V: Distribution of C-kit immunoreactivity according to histological type and survival in rhabdomyosarcoma cases.

		Number of Cases (%)	C-kit Pozitive (%)	C-kit Negative (%)
Histological Type	**Alveoler**	12 (48)	6 (40)	6 (60)
	Embryonal	9 (36)	7 (46.6)	2 (20)
	Spindle Cell	2 (8)	1 (6.7)	1 (10)
	Pleomorphic	1 (4)	1 (6.7)	-
	Botryoid	1 (4)	-	1 (10)
	Total	**25 (100)**	**15 (100)**	**10 (100)**
Survival	**Live**	12 (86)	8 (89)	4 (80)
	Dead	2 (14)	1 (11)	1 (20)
	Total	**14 (100)**	**9 (100)**	**5 (100)**

Figure 1A: Cytoplasmic c-kit positivity in osteosarcoma (x200).

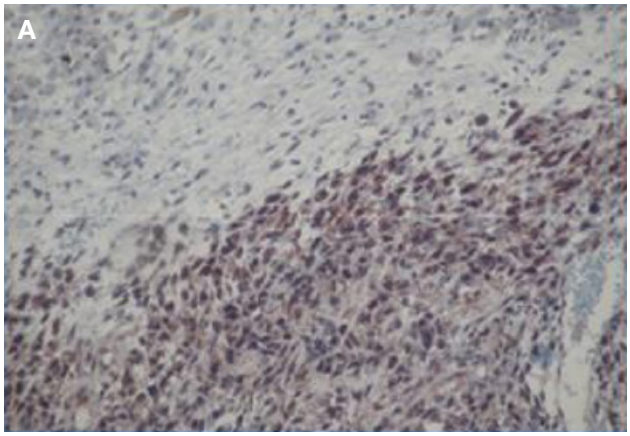


Figure 1B: Cytoplasmic c-kit positivity in Ewing sarcoma (x200).

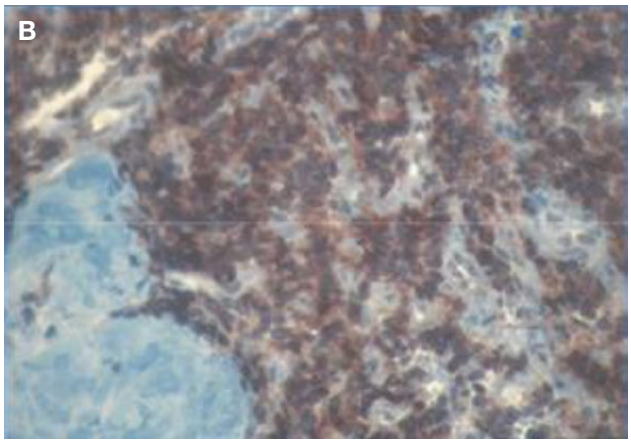


Figure 1C: Strong positivity of cytoplasmic-membranous c-kit in rhabdomyosarcoma (x400).

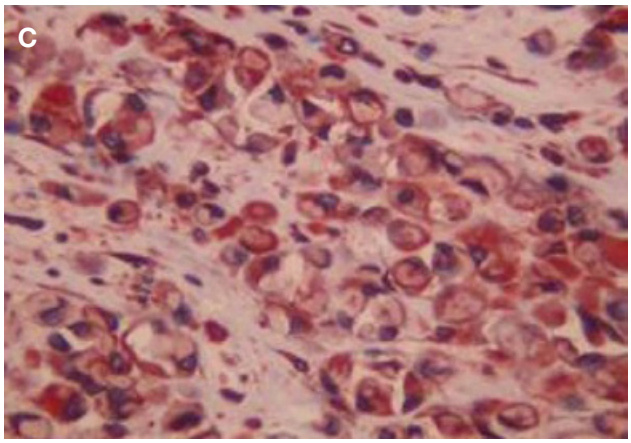


Figure 1D: Strong cytoplasmic-membranous c-kit positivity in rhabdomyosarcoma (x200).

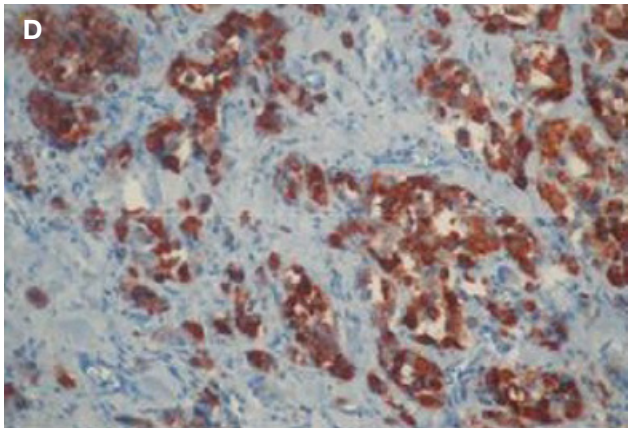
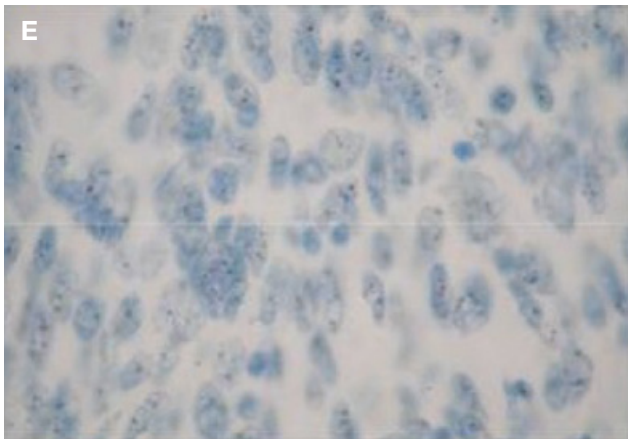


Figure 1E: Increased number of signals in c-erb-B2 gene by SISH method in rhabdomyosarcoma (x400).



Discussion

With the demonstration of the roles of c-kit and c-erb-B2 in the etiopathogenesis of some epithelial and stromal tumors, and the successful results of treatments targeting these receptors in recent years, the expression of c-kit and c-erb-B2 has been investigated in many different tumors.

Mutations inc-kit have been identified in tumor pathogenesis for more than 20 years, and nearly 500 different mutations have been revealed over the years. More than 80% of small cell carcinoma of the lung, malignant melanoma, colorectal cancer, and gastrointestinal stroma are among these tumors[15].

Although c-kit is a transmembrane receptor protein, its expression is usually cytoplasmic by immunohistochemistry in sarcomas. In a study by Sabah et al., it was explained that the distinction between cytoplasmic and membranous staining was difficult in tumors with a high nucleocytoplasmic ratio. Therefore, both membranous-cytoplasmic and membranous staining were considered positive[16,17]. In studies investigating c-kit expression in OS, the positivity rate obtained by Entz-Werle et al.[7] was 57%, by Miiji et al.[18] was 46%, and by Saeter et al.[19] was 62.5%, whereas we obtained a positivity rate of 44% in our study. In our study, it was determined that the overall survival was lower in cases with c-kit expression, and this findingwas statistically significant (p=0.04).While the 5-year survival was 100% in negative cases in a study by Werle et al.[7] and 85% in positive cases, it was reported that c-kit expression could be a poor prognostic parameter, which is consistent with our study. In a series of 100 cases reported by Sulzbacher et al.[20], 20% c-kit positivity was detected,and there was no correlation withoverall survival. It has been suggested that c-kit expression can be observed in OS, but that it has no effect on prognosis[21].

Studies on c-kit expression in soft tissue sarcomas have reported different results in the literature. C-kit expression was detected in between 38% and 71% of tumors in the ES tumor family[22]. In our study, positive staining was detected in 44% of the ES cases. The mean survival was 13 months in patients with c-kit expression and 41 months in patients without c-kit expression; however, remarkably, no statistically significant correlation was found between c-kit positivity and overall survival (p>0.05). Since no relationship could be established with survival in ES cases with c-kit expression, other clinicopathological parameters were thought to be more determinative of survival.

In different series investigating c-kit expression in RMS cases, expression rates were found to be quite low in the literature. Landuzzi et al. reported that c-kit expression is negligible in RMS, but stem cell factor production is quite high[23]. In another study, c-kit was found to be positive in 6% of 105 RMS cases. In contrast tothe literature, we found c-kit positivity in 60% of the cases in the RMS group. The average survival time was 35 months in positive cases and 43 months in negative cases. However,there was no statistically significant relationship between c-kit staining and survival (p>0.05)[24].

Overexpression of c-erb-B2 protein due to the amplification of the *HER2* gene has been identified in many tumors originating from breast, ovary, lung, pancreas, stomach, colon, salivary gland, bladder, and kidney. C-erbB2 expression has been considered to promote increased tumorigenesis and metastatic potential, as well as resistance to chemotherapeutic agents[11,25,26].

In a case series by Onda et al.[25] in which 26 cases of OS were examined, c-erb-B2expression was detected at a rate of 42%, while gene amplification and mutation were not observed.Another similar study[27] suggested a statistically significant correlation of c-erb-B2 expression with multiple metastases and recurrences in a series of 115 OS cases, including primary tumor biopsies and resections for metachronous lung cancers. In our study, staining with c-erb-B2 was not observed in any of the 25 OS cases;only nonspecific cytoplasmic staining was detected in the muscle tissue adjacent to the tumor in four cases. It is thought that the conflicting results of studies on c-erb-B2 expression in OS may be due to the use of different fixation and decalcification methods, antibody clones, antigen retrieval methods, and evaluation scores[15].

In studies investigating c-erb-B2expression in ES, cytoplasmic c-erb-B2staining was found at a rate of 16% by Scotlandi et al. and 27% by Thomas et al.[26,27], but no correlation with clinical parameters was found. In our ES study group consisting of 25 cases, c-erb-B2protein expression was not observed.

In studies investigating c-erb-B2 expression in RMS cases, there are series that show 9%–33% positivity, as well as series in which all of the cases were negative. However, no correlation was found between clinical parameters and c-erb-B2 expression in cases with positive staining[24,28]. In our study, positive staining with theanti-c-erb-B2antibody was found in 7 (28%) of RMS cases, similar to the rates in some studies in the literature, but *HER2* gene amplification was found in only 1 of these cases—a remarkable finding. It is known that expression of c-erb-B2 protein in tumors by IHC does not imply a specific alteration in the *HER2* gene in all cases, so we believe that the results should be supported by molecular tests.Notably, there was no statistically significant correlation between c-erb-B2 immunoreactivity and survival (p>0.05).

In our series in which cases of common bone and soft tissue tumors were evaluated,we concluded that c-kit and c-erb-B2 expression did not have prognostic significance in ES and RMS, but c-kit expression in OS isa poor prognostic parameter. The different results observed in studies on c-kit and c-erb-B2 expression may be due to antibody clones used, IHC methods used, and evaluation criteria. By using standardized IHC methods such as those used for breast cancer, stomach cancer, and gastrointestinal stromal tumorsin studies, as well as investigating gene mutations usingmolecular methods and comparing and sharing the results along with follow-ups inlong-term and large patient series, it will be possible to accurately determine the contextsin which molecular therapies targeting these receptors could be used and achieve meaningful results for patients.

Competing interests

All authors declare no competing interest.

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