The expression of CRM1 is associated with prognosis in human osteosarcoma

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Abstract. The nuclear export protein chromosomal region maintenance/exportin 1/Xpo1 (CRM1) is involved in the nuclear export of proteins and messenger RNAs and, thus, mediates the subcellular distribution of important molecules. Osteosarcoma is a ubiquitous and highly aggressive malignant bone tumor. The expression of CRM1 protein in human osteosarcoma has not been reported to date. We investigated the expression of CRM1 in 57 human osteosarcoma and 5 normal cartilage tissues. Western blot investigation revealed expression of CRM1 was significantly increased in osteosarcoma compared with normal tissues. High expression of CRM1 was significantly associated with increased serum level of alkaline phosphatase (ALP, P=0.001) but did not associate with that of lactate dehydrogenase (LDH, P=0.06). In univariate analysis, a significant association between CRM1 expression and tumor size (P=0.014) as well as histological grade (P=0.003) was observed, while high CRM1 expression was not correlated with the other clinicopathological parameters. In Kaplan-Meier survival analysis, high CRM1 expression was a significant prognostic indicator for progression-free survival (P=0.016) as well as overall survival (P=0.008). Multivariate analysis demonstrated that expression of CRM1 was an independent prognostic parameter for longer overall survival (95% CI, 1.27-5.39). Additional prospective studies are required to investigate the prognostic role of high expression of CRM1.

Introduction

Osteosarcoma is the most frequent primary malignant bone tumor. It most often originates in the metaphyses of long bones of adolescents. Due to the high rate of systemic spread, cure is rare after surgical treatment alone. The inclusion of aggressive polychemotherapy into an interdisciplinary treatment concept has led to dramatic prognostic improvements in young patients with seemingly localized extremity disease, with relapse-free survival rates of ~50-80% reported by specialized centers or multicentric groups (1-8). However, a substantial subgroup of all osteosarcoma patients lacks prognostic markers that could distinguish patients before therapy and drive treatment choices. Consequently, considerable uncertainty about the true success rate of osteosarcoma treatment in the era of polychemotherapy remains. Some research has been made to evaluate new prognostic markers that could contribute to the choice of treatments. Nevertheless, selecting novel prognostic marker is necessary for further improvement in treatment of osteosarcoma (9,10).

Several oncogenes and tumor suppressor genes are reported to be involved in oncogenesis of OS. Although survival rate increased up to 60-70% within the last 20 years, the problem of non-response to chemotherapy remains. There are many factors thought to have an influence on prognosis of osteosarcoma. The data from some researchers demonstrate that in osteosarcoma, pretreatment serum alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) levels have a prognostic value and they should be considered when evaluating different therapeutic protocols and in planning new randomized clinical trials. It is known that many patients with osteosarcoma have high serum ALP and LDH levels (11,12). However, the exact role of these factors is still controversial.

The human nuclear export protein chromosomal region maintenance/exportin 1/Xpo1 (CRM1) mediates the nuclear export of proteins and messenger RNAs and, thus, is an important regulator of subcellular distribution of key molecules (13-18). In addition to cell-biologic studies that suggested a fundamental role for CRM1 in the regulation of mitosis, Noske *et al* reported that the expression of this protein is a prognostic factor in human ovarian cancer (19). However, more data should be obtained from other cancers to further evaluate the prognostic value of CRM1 expression.

In this study we investigated the expression of CRM1 and evaluated its prognostic value in osteosarcoma. The

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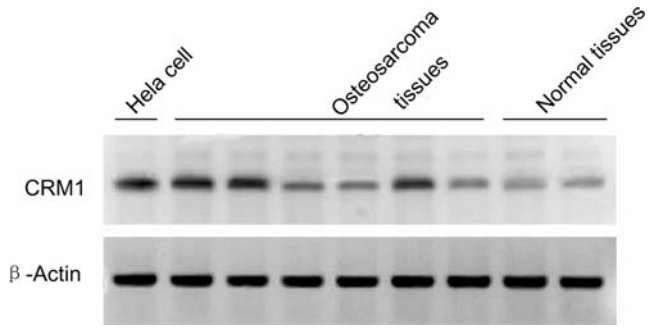

Figure 1. Western blot investigation of CRM1 expression in osteosarcoma and normal tissues. Hela cell lysates is used as positive control. Total protein (200 μg) is loaded in each lane. Densitometric ratio (CRM1 expression in tumor or normal sample vs. that in Hela cell) >0.5 is considered as high expression, ≤0.5 as low expression.

correlation between CRM1 expression and the clinico-pathological parameters of osteosarcoma, including LDH and ALP, was also analyzed.

Materials and methods

A total of 57 patients were studied. All samples were preoperative samples obtained by biopsy. Clinical and radiological examination revealed the suspicion of osteosarcoma, and then the definitive pathological diagnosis of osteosarcoma was made by the CT-guided puncture or incisional biopsy. There were 57 admitted patients of limb osteosarcoma from January 2002 to December 2006 in Department of Oncology, The Affiliated Sixth People's Hospital, Shanghai Jiaotong University. Pathological grades: G1, well-differentiated; G2, moderately differentiated; G3, poorly differentiated and G4, undifferentiated. The patients were followed up after diagnosis. Physical examination was performed every 3 months in all patients for the first 2 years and then twice/year. Radiographic studies (limb local and chest X-ray), and liver ultrasounds were performed every 12 months; bone scans and computed tomography scans were performed whenever clinically indicated. Blood tests, including electrolyte and liver function profiles and complete blood cell counts, were repeated at every follow-up visit. Pregression-free survival (PFS) was calculated as the period from surgery until the date of the first recurrence.

Treatment. Drugs: methotrexate (MTX), cisplatin (DDP), ifosfamide (IFO) and epirubicin (EPI). Thirty-one cases received 3 courses of neoadjuvant chemotherapy, among them, further 14 cases received one course of chemotherapy with arterial infusion of DDP and EPI. Post-chemotherapeutic surgically treated patients include limb salvage 27 cases, amputation 10 cases. Each post-operative case received 3-8 courses of adjuvant chemotherapy of equal courses. Among 26 cases who did not received neoadjuvant chemotherapy, 13 cases with limb salvage, 13 cases with amputation, post-operative cases with 4-8 course of adjuvant chemotherapy with average courses.

Table I. Patient demographics.

		No. of Patients (N=57)
Age (years)		
Median	23.2	
Range	5-57	
<12		11
≥12		46
Gender		
Male		34
Female		23
Local treatment		
Surgery, amputation		23
Surgery, limb-salvage		27
Surgery, unknown		7
History of symptoms		
Median (days)	72	
<70 days		25
≥70 days		32
Tumor size (portion of whole bone)		
< one third		27
≥ one third		30
Metastasis		
Absent		44
Detected		13
Histological type		
General		46
Dilated blood vessels		5
Cortex		3
Intra-medullary high differentiated		1
Others		2
Grade		
G1		32
G2		17
G3		6
G4		2
Primary location		
Lower segment of femur		28
Upper segment of tibia		17
Upper segment of humerus		5
Upper end of fibula		3
Upper segment of femur and lower segment of tibia		2
Lower segment of humerus and fibula		2
Duration follow-up (months)		
Median	32.5	
Range	10-52	
Relapse		18
Death		22

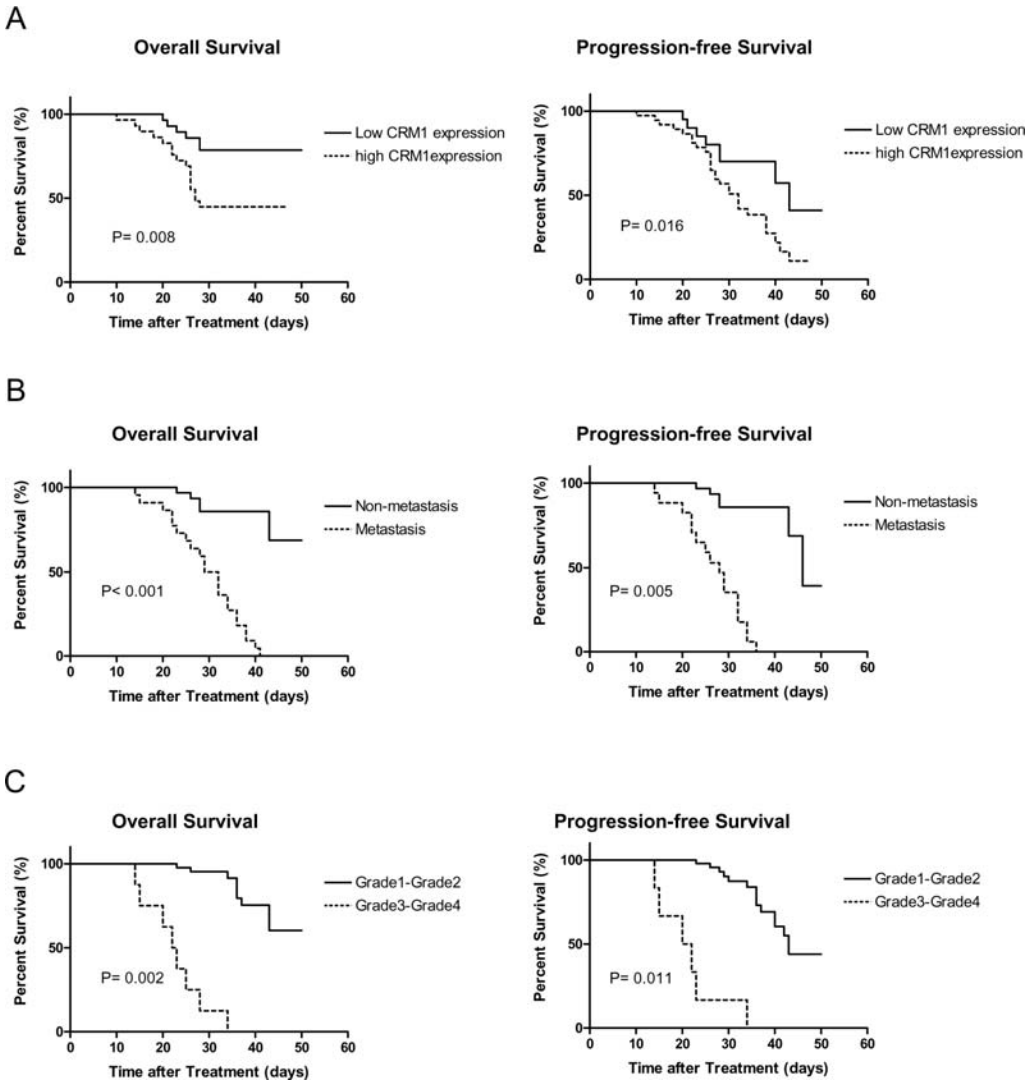

Figure 2. Kaplan-Meier survival analysis of overall survival and progression survival of (A) CRM1 expression, (B) primary metastasis and (C), pathological grades.

Western blotting. For Western blotting, samples were homogenized in a solution of 10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin at pH 7.9. Protein extracts were then subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The monoclonal antibody against CRM1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The membrane was incubated with primary and secondary antibodies, respectively, for 1 h at room temperature. Signals were developed by ECL Kit (Amersham Pharmacia Biotech Inc., Buckinghamshire, UK). Hela cells express CRM1 protein and were used as control (20). Blot quantitation was done with a Molecular Dynamics Laser Densitometer (Model PSD) and the Image Quant Version 1 software (21).

Statistical analysis. The statistical significance of the correlation between expression of CRM1 and several clinicopathological parameters was assessed by Fisher's exact test, χ^2 test, or χ^2 test for trends as indicated. The probability of overall survival as a function of time was determined by the Kaplan-Meier method and the log-rank test. Multivariate survival analysis was performed using the Cox regression model. P<0.05 were considered as significant. For the statistical evaluation, the SPSS software version 12.0 was used (SPSS, Inc., Chicago, IL).

Table II. CRM1 expression in ostoesarcomas and normal tissues.

	Osteosarcomas (n=57)	Normal tissues (n=5)	P-value (Fisher's exact test)
CRM1 expression			0.037
Low	28	5	
High	29	0	

Results

Clinicopathological characteristics of patients with osteosarcoma. The basic demographics of this group and the

Table III. Relationship of high CRM1 expression with serum LDH, ALP and other clinicopathological factors in patients with osteosarcomas.

Characteristic	All cases	CRM1 normal (%)	CRM1 increased (%)	P-value
Serum LDH (n=57)				0.065
Normal	44	24 (54.5)	20 (45.5)	
Increased	13	4 (30.8)	9 (69.2)	
Serum ALP (n=57)				0.002
Normal	18	13 (72.2)	5 (27.8)	
Increased	39	15 (38.5)	24 (61.5)	
Gender				0.06
Male	34	20 (58.8)	14 (41.2)	
Female	23	8 (34.8)	15 (65.2%)	
History of symptoms				0.22
<70 days	25	14 (56.0)	11 (44.0)	
≥70 days	32	14 (43.8)	18 (56.2%)	
Tumor size				0.014
< one third	31	15 (48.4)	16 (51.6)	
≥ one third	26	13 (50.0)	13 (50.0)	
Metastasis				0.84
M_0	44	22 (50.0)	22 (50.0)	
M_1	13	6 (46.2)	7 (53.8)	
Histological grade				0.003
G1	32	21 (65.6)	11 (34.4)	
G2	17	6 (35.3)	11 (64.7)	
G3	6	1 (16.7)	5 (83.3)	
G4	2	0	2	
Age (years)				0.32
<12	11	6 (54.5)	5 (45.5)	
≥12	46	22 (47.8)	24 (52.2)	
Necrosis rate post chemotherapy				0.86
<90%	30	15 (50.0)	15 (50.0)	
≥90%	27	13 (48.1)	14 (51.9)	

pathological characteristics are shown in Table I, respectively. The median age was 23.2 years (range 5-57). Most of patients were in the range 12-25 years. Twenty-seven (47.4) patients obtained limb salvage surgical treatment, 18 cases had a relapse and 22 died during the follow-up.

CRM1 protein expression in human osteosarcoma is increased compared to that in normal tissues. We determined expression of CRM1 protein in tumor tissues of 57 cases of osteosarcoma patients and in normal tissues of 5 cases, non-tumor patients, by Western blotting. As shown in Fig. 1, compared with the low CRM1 expression in normal tissues, the CRM1 expression in osteosarcoma tissues showed obviously increased levels. There were 29 patients with high CRM1 expression of the total 57 patients, and none of the 5 normal tissues expressed increased CRM1 (Table II). This significant difference of CRM1 expression in osteosarcomas and normal tissues (P=0.037) suggested that CRM1 might be a prognostic factor in osteosarcoma.

High CRM1 expression related with some clinicopathological factors in patients with osteosarcomas. We investigated the expression of CRM1 by Western blotting (Fig. 1) and analyzed the correlation between high expression of CRM1 and clinicopathological factors in patients with osteosarcomas, including serum ALP and LDH. Our data demonstrated that the increased expression of CRM1 was significantly associated with increased serum level of ALP (P=0.001) but did not associate with that of LDH (P=0.065). There was also a significant association

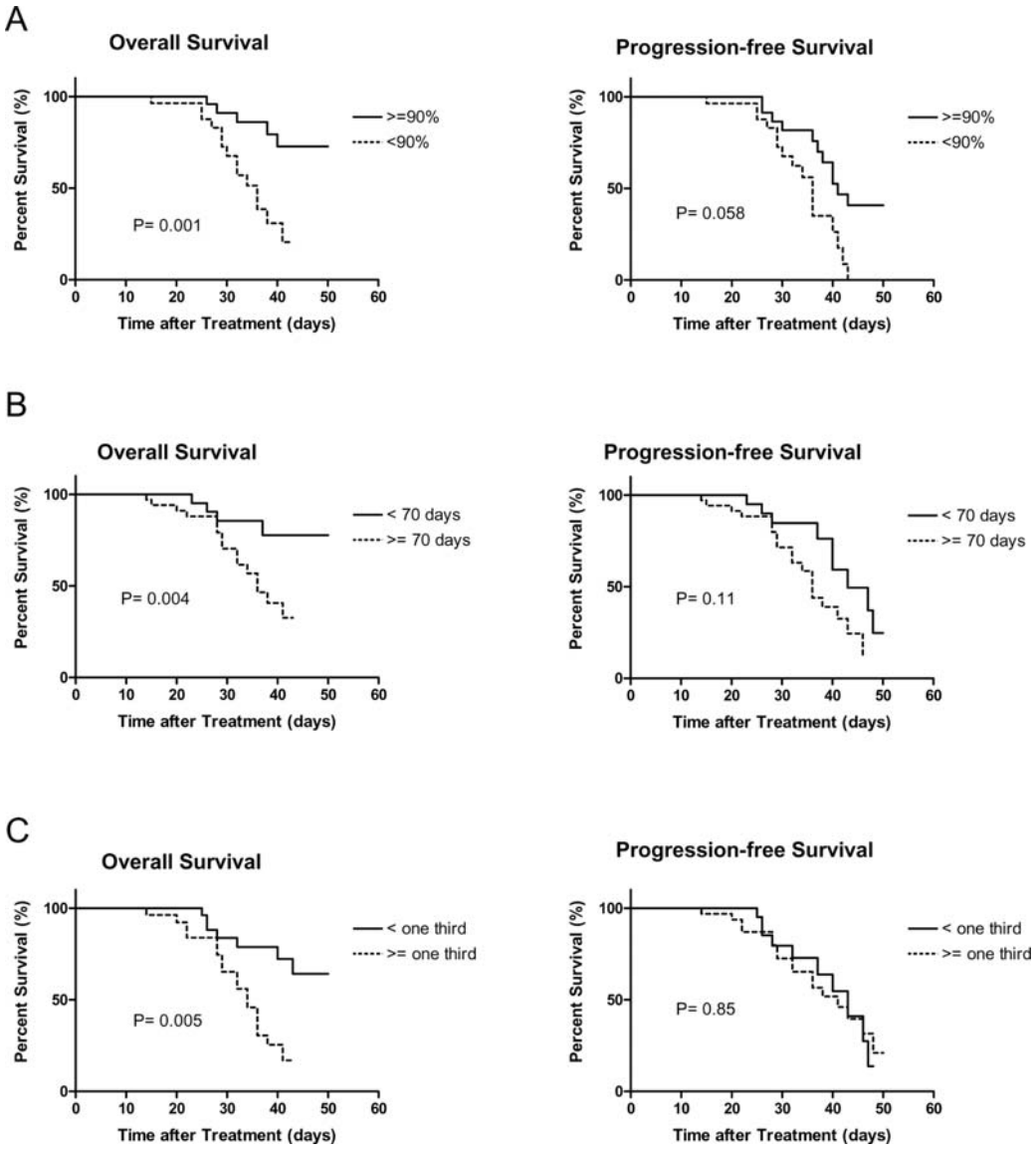

Figure 3. Kaplan-Meier survival analysis of overall survival and progression survival of (A) necrosis rate post-chemotherapy, (B) history of symptom and (C) tumor.

between CRM1 expression and tumor size (P=0.014) as well as histological grade (P=0.003), while expression of CRM1 was not correlated with gender, tumor size, metastasis or age (Table III).

High CRM1 expression is a prognostic factor for overall and progression-free survival in univariate Kaplan-Meier analysis. In Kaplan-Meier survival analysis, high expression of CRM1 was a significant prognostic indicator for overall (P=0.008) as well as progression-free survival (P=0.016) (Fig. 2). In addition, the presence of metastases and histological grade were significant prognostic indicators for overall and progression-free survival (Fig. 2). Necrosis rate post-chemotherapy, history of symptoms and tumor size were significant prognostic indicators for overall survival but not for progression-free survival (Fig. 3).

Multivariate survival analysis. Results by multivariate survival analysis demonstrated that high CRM1 expression, increased serum ALP, the presence of metastasis, worse histological grade and <90% tumor necrosis were significantly related to shorter survival (Table IV).

Discussion

Osteosarcoma is the most frequent malignant tumor of childhood, excluding hematopoietic malignancy (22). It usually occurs in patients between 10 and 25 years of age, which suggests an abnormality in the growing bone cells that leads to malignant disease. In addition, osteosarcoma is slightly more common in tall children and in males (23,24). Most osteosarcoma arising are located in the metaphyseal area of the long bones, particularly, lower end of femur, the upper end of tibia and the upper end of the humerus. Various prognostic factors are tumor size, response to chemotherapy, post-chemotherapy tumor necrosis, and serum elevation of alkaline phosphatase levels. Metastases at diagnosis were present in 23% of our patients, which is higher than the

Table IV. Multivariate survival analysis (Cox regression model) for overall survival of 57 patients with osteosarcomas.

Characteristic	RR (95% CI)	P-value
CRM1 expression		
High	1.00	
Low	2.83 (1.27-5.39)	0.006
Serum LDH (n=57)		
Normal	1.00	
Increased	2.39 (0.85-6.72)	0.05
Serum ALP (n=57)		
Normal	1.00	
Increased	2.52 (1.32-4.55)	0.007
Gender		
Male	1.00	
Female	2.62 (0.85-6.72)	0.098
History of symptoms		
<70	1.00	
≥70	2.83 (0.45-4.36)	0.13
Tumor size		
< one third	1.00	
≥ one third	2.45 (1.22-5.19)	0.06
Metastasis		
M_0	1.00	
M_1	2.29 (1.65-3.92)	0.003
Histological grade		
G1-G2	1.00	
G3-G4	2.41 (1.56-5.75)	0.003
Age (years)		
<12	1.00	
≥12	1.51 (0.67-3.41)	0.32
Necrosis rate post Chemotherapy		
<90	1.00	
≥90	2.54 (1.52-5.63)	0.005

RR, relative risk; CI, confidence interval.

frequency reported by other investigators (25-27). This clinical characteristic reflects the frequency of advanced disease at diagnosis among our patient population. We then documented the possible reasons for this characteristic. The significant correlation of the presence of metastases with the time from onset of symptoms to diagnosis, tumor size, or pathological grade has been demonstrated. It was reported that primary tumor spread was associated with shorter pre-diagnostic symptom duration and hypothesized that this could be related to a more aggressive biologic behavior (28). In our studies, the history of symptoms, the presence of metastases, pathological grades and tumor size were identified as the significant factors influencing outcome. Among them, the history of symptoms is an improvable clinical factor. By establishing a collaborated group of diagnosis and treatment of osteosarcoma in China, we can educate the potential patients and doctors to be alert to the symptoms of this disease and may therefore increase the rate of early diagnosis. Shortening the time from the set of symptoms to diagnosis may benefit the prognosis of the patients in China.

Several studies have demonstrated that CRM1 is important in nuclear-cytoplasmic transport and control of mitosis. Regarding the transport function, CRM1 is the main nuclear export receptor in humans (29-33). Mechanistic studies have demonstrated that some important molecules related to tumor biology depend on the CRM1 nuclear export pathway (34-37). It has also been demonstrated that CRM1 functions in complex with ras-related nuclear protein guaosine triphosphatase (RAN-GTPase) to control several processes during cellular mitosis. Therefore, CRM1 may associate with carcinogenesis.

In this study, we investigated the expression of the human CRM1 protein in osteosarcoma and in normal tissue. To our knowledge, this is the first study investigating expression of CRM1 in osteosarcoma and second study in cancer (19). The expression of CRM1 was higher in osteosarcomas compared with that in normal tissues. These tumors showed a significantly higher mitotic rate and were poorly differentiated. Patients with osteosarcomas showing increased CRM1 expression had a reduced progression-free and overall survival rate.

The cellular mechanisms responsible for the worst prognosis of tumors with an increased expression of CRM1 are still unknown. Our statistical analysis, suggests a link between overexpression of CRM1 and increased serum level of ALP. It is also supported by our analysis that there is an association between high CRM1 expression and worse pathological grade. Since ALP expression is not correlated with differentiation, it is possible that another CRM1 target is involved, which is important for cell differentiation. Additional studies will be required to analyze the molecules related to cell differentiation that are regulated by CRM1 in osteosarcoma.

It should be emphasized that the present study is a retrospective study with some limitations. In our study group, data on therapy as well as intraoperative residual tumor were retrospectively not available for all patients and could therefore not be included in the multivariate analysis. Additional large-scale prospective and retrospective studies are needed to investigate whether CRM1 expression is indeed of practical utility as a prognostic predictor.

Generally, when the determination of the immunoreactive pattern of CRM1 expression is combined with other clinicopathological factors, it may improve the prognostic evaluation of osteosarcoma patients. The CRM1 is a prognostic factor in human osteosarcoma and may contribute to identification of individuals who are at high risk for poor survival.

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