Expression of MSH2 and MSH6 on a Tissue Microarray in Patients with Osteosarcoma

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Abstract. *Background/Aim: Reliable prognostic factors for the outcome of patients with osteosarcoma (OS) remain elusive. We analyzed the relationship between immunohistochemical expression of deoxyribonucleic acid (DNA) mismatch repair (MMR) proteins, MutS protein homolog 2 (MSH2) and MSH6 using a tissue microarray (TMA) with respect to OS patient demographics and survival time. Materials and Methods: We retrieved tumor tissue specimens from bone tissue originating from surgical primary tumor specimens of OS patients to generate a TMA and stained sections with antibodies against MSH2 and MSH6. Results: Tumor resections of 67 patients with a mean follow-up of 98 months were evaluated. MSH2 was expressed in nine (13%), MSH6 in ten (15%) and combined MSH2 and MSH6 (MSH2/6) in six (9%) patients. Significantly shorter survival times were associated with expression of MSH6, MSH2/6, as well as simultaneous non-response to chemotherapy and presence of metastasis. Conclusion: The survival time of patients with OS may be predicted by local expression of MSH6 and MSH2/6 in surgical primary tumor resections. This study shows the prognostic value of the local expression of DNA MMR proteins, as markers for poor prognosis of OS patients.*

Osteosarcoma (OS) is the most common primary malignant bone tumor (1) and its incidence peaks at adolescence (2). Males are slightly more frequently affected than females (3). The metaphysis of the long bones of the extremities is more commonly affected than the axial skeleton of the pelvis, spine and head (4, 5). Following neoadjuvant chemotherapy, surgical tumor resections provide tissue specimens for the assessment of tumor necrosis rate, histology, grading and evaluation of tumor biomarkers before further adjuvant chemotherapy may be initiated (6-8). Despite advances in therapeutic approaches, five-year overall survival rates have been reported as 78% (9) and are usually lower for non-responders to chemotherapy (10) and patients with metastasis (11, 12).

Albeit the limited number of studies (8, 13-17) on tumor biomarkers, these are important for treatment decisions, patient discrimination regarding the response to chemotherapy and the incidence of metastasis, prediction of patient survival times and patient education (18-21). Deficiencies in mismatch repair (*MMR*) genes have been associated with various cancers, such as hereditary non-polyposis colorectal cancer (HNPCC) (22-25), as well as sarcomas (26, 27). Furthermore, the expression of MSH2 on OS cells has been suggested to potentially play a role in resistance to chemotherapeutic agents (28). Importantly, a recent case report has described an association between mutations in the *MSH2* and *MSH6* genes and OS for the first time (29). However, the potential association of MSH2 and MSH6 has not been investigated in a series of patients.

The MMR system of erroneous deoxyribonucleic acid (DNA) replication is an essential pathway in all living cells (30). There are nine human *MMR* genes (31), with MutS homolog 2 (MSH2) and MutS homolog 6 (MSH6) being key proteins. Together, they form a heterodimer, MutSα, in which MSH2 stabilizes MSH6, which possesses a nuclear localization sequence (NLS) (32-34). In the nucleus, mispaired bases or insertion-deletion loops (35-37) are recognized and signaled by the MutS/DNA complex (38). The MutSα heterodimer associates with MutL homolog 1 (MLH1) to form MutLα. In turn, this complex recruits an exonuclease, which ultimately leads to removal of parts of the DNA strand containing the wrongly-incorporated base by excision repair.

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Because OS biomarkers remain barely known for patient discrimination concerning non-response to chemotherapy, metastasis and unfavorable survival times, the aim of the present study was to investigate the demographics and survival times of OS patients with regard to local expression of MSH2 and MSH6 on primary patient material.

Materials and Methods

We retrieved tumor tissue specimens from bone tissue originating from surgical primary tumor resections of patients diagnosed with OS between December 1987 and October 2005 (39). A retrospective analysis of patient data was performed and age, gender, tumor location, tumor type, response to chemotherapy, local recurrence, metastasis and patient survival were documented. The histopathological classification by the World Health Organization (WHO) (3) was used to group tumor tissue specimens and the cooperative osteosarcoma study (COSS) protocol (40) was followed for chemotherapy. Response to chemotherapy was evaluated according to Salzer-Kuntschik (41) where less than 10% of vital tumor tissue indicated a response to chemotherapy. The study complies with the regulations of the local ethics committee.

A useful tool for the immunohistochemical examination of many different tumor biomarkers in small amounts of tissue of patients is a tissue microarray (TMA) (17, 39, 42, 43). Therefore, biopsies of non-necrotic tumor tissue were collected from surgically resected OSs (39).

A semi-automatic punch machine with a hollow needle (diameter of 0.6 mm) removed two to six tissue cores (spots) from the biopsies (14, 44), which were subsequently implanted into a paraffin block in an array pattern by a computer-operated electromotor resulting in 174 spots concerning surgical resections. A total of 404 spots (Figure 1A) were present on the entire TMA because tissue specimens from presurgical biopsies, recurrences and metastases originating from several patients were implanted on the TMA. Two spots are sufficient to represent an entire biopsy in 95% of cases (45). Sections of 2 μm were cut into the paraffin block and moved to an adhesive-coated slide system (Instrumedics, Hackensack, NJ, USA), de-paraffinized and processed with an automated staining system (Ventana Medical Systems, Tucson, AZ, USA). An ethylenediaminetetraacetic acid (EDTA)-containing buffer (Bond Epitope Retrieval Solution 2, Vision BioSystems, Wetzlar, Germany) was used for antigen retrieval. Antibodies against MSH2 (dilution 1:120) (Novocastra, Stockholm, Sweden by Leica Microsystems, Wetzlar, Germany) and MSH6 (dilution 1:200) (Becton, Dickinson and Company Biosciences, Allschwil, Switzerland) were used for antigen staining and hematoxylin was added for counter-staining. Antibody reactions resulted in a brown product, which was visualized by iVIEW DAB Kit (Ventana Medical Systems). For quality control, we used three formalin-fixed paraffin-embedded control sections with a known staining pattern.

A physician was trained by a pathologist and performed grading independently from a student who used a MATLAB analysis tool based on color deconvolution (46). Both investigators were blinded to clinical information. Similar grading was found in more than 95% of cases and a consensus was found for the remainder. Only viable tissue cores were scored and graded according to a three level scale. Negative staining with <10% of stained cells with no or weak staining intensity was defined as grade 1 (Figure 1B). Positive staining with ≥10% stained cells with weak staining intensity was defined as grade 2 (Figure 1C) and strong, maximum staining intensity was defined as grade 3 (Figure 1D) (47). Negative staining (grade 1, no expression) was compared to positive staining (grades 2 and 3, expression).

Patient survival was calculated by the Kaplan-Meier curves. Statistical differences between groups were measured with the log-rank (Mantel-Cox) test. *p*-Values <0.05 were considered statistically significant. GraphPad Prism 5.01 software (GraphPad Software, La Jolla, CA, USA) and SPSS statistics v21.0 (IBM Corp., Armonk, NY, USA) were used.

Results

Sixty-seven OS patients (39) were investigated with regard to demographic parameters (Table I), as well as immunohistochemical expression of MSH2 and MSH6. The mean age was 22 years (range, 2 to 66) and there were 24 (36%) female and 43 (64%) male patients. The mean follow-up time was 98 (range=7-213) months and the mean 5-year survival rate was 73%. At last follow-up, 46 (69%) patients were alive. Our study included 60 (90%) patients <40 years and 7 (10%) patients ≥40 years; the same patient cohort can also be equally distributed into 31 (46%) patients <18 years and 36 (54%) patients ≥18 years (48). The OS was located in an extremity in 52 (78%) patients and an axial location of the tumor (pelvis, spine or head) was found in 15 (22%) patients. We also found various OS types, namely 46 (69%) osteoblastic, 11 (16%) chondroblastic, 6 (9%) fibroblastic and 4 telangiectatic (6%) tumors. Twenty-three (34%) patients did not respond to chemotherapy, whereas 30 (45%) patients were responders. For the remaining 14 (21%) patients no information about the response upon neoadjuvant chemotherapy was available. Local recurrence was found in 9 (13%) patients with a mean of 31 (range=8-111) months. Metastases were detected in 24 (36%) patients. Metastases were present at the time of diagnosis in 5 (7%) patients. Metastases developed in the lung in 20 (30%) patients, in both the lung and bone in three patients (4%) and at an unknown site in one patient (1%).

Kaplan-Meier survival analyses did not show any significant differences for gender (p=0.808) or tumor type (p=0.345). Patients <18 years did not have significantly (p=0.892) different survival times compared to patients ≥18 years, there was a significantly (p=0.003) shorter survival time of 65 (95% confidence interval (CI)=19-110) months for patients ≥40 years compared to a mean survival time of 164 (95% CI=143-184) months for patients <40 years (Figure 2A). Patients with axial tumors of the pelvis, spine or head displayed a significantly (p=0.001) shorter survival time of 85 (95% CI=48-123) months than patients with tumors of the extremities where the mean survival time was 173 (95% CI=152-193) months (Figure 2B). Not surprisingly, patients with local recurrence of the tumor had a significantly (p<0.0001) shorter survival time of 66 (95% CI=32-100) months than patients without local recurrence where the mean survival time was 171 (95% CI=151-191) months (Figure 2C). Non-responders to chemotherapy survived

Table I. *Patients' survival and p-values are shown for gender, age, tumor type, tumor location, local recurrence, response to chemotherapy and metastasis. There were two age groups[1, 2] due to the uneven patient distribution in the more commonly used first age group[1].*

					p-Value
Gender	Females		Males		0.808
Patients	n=24		n=43		
Survival time (months)	150 (115-186)		152 (126-177)		
5-year survival	65%		76%		
Age[1]	<40 years		≥40 years		0.003
Patients	n=60		n=7		
Survival time (months)	164 (143-184)		65 (19-111)		
5-year survival	29%		78%		
Age[2]	<18 years		≥18 years		0.892
Patients	n=31		n=36		
Survival time (months)	149 (119-179)		156 (128-184)		
5-year survival	71%		74%		
Location	Extremity		Axial		0.001
Patients	n=52		n=15		
Survival time (months)	85 (48-123)		173 (152-193)		
5-year survival	81%		43%		
Type	Telangiectatic	Chondroblastic	Fibroblastic	Osteoblastic	0.345
Patients	n=4	n=11	n=6	n=46	
Survival time (months)	79 (52-106)	115 (64-166)	122 (78-166)	165 (141-188)	
5-year survival	75%	60%	67%	76%	
Response to chemotherapy	Non-responders		Responders		0.008
Patients	n=23		n=30		
Survival time (months)	99 (70-129)		180 (157-202)		
5-year survival	52%		87%		
Local recurrence	Recurrence		No recurrence		<0.0001
Patients	n=9		n=58		
Survival time (months)	66 (32-100)		171 (151-191)		
5-year survival	44%		77%		
Metastasis	Metastasis		No metastasis		<0.0001
Patients	n=24		n=43		
Survival time (months)	70 (41-100)		200 (185-214)		
5-year survival	33%		95%		

Survival: mean (95% CI), p-Value were calculated by the long-rank test.

for a mean time span of 99 (95% CI=70-129) months, which was significantly (p=0.008) shorter than responders to chemotherapy who survived for a mean time span of 180 (95% CI=160-202) months (Figure 2D). The mean survival time of 70 (95% CI=41-100) months for patients with metastasis was significantly (p<0.0001) shorter than the survival time of 200 (95% CI=185-214) months for patients without metastasis (Figure 2E). The 5-year survival time for patients with metastasis was only 33%, compared to 95% for patients without metastasis.

With regard to MSH2, positive staining on a TMA was found in nine (13%) OS patients and negative staining was seen in 58 (87%) patients. Kaplan-Meier survival analysis did not show an association between survival time and immunohistochemically-detectable expression of MSH2 (p=0.292) (Figure 3A) (Table II). There was also no association (p=0.063) between expression of MSH2 and non-responders to chemotherapy (Figure 3B, Table III). Yet, the mean survival times were significantly (p<0.0001) shorter for patients with expression of MSH2 and metastasis (mean survival time of 17 (95% CI=7-27) months, 5-year survival rate of 0%) than the mean survival times for patients without expression of MSH2 and no metastasis (203 (95% CI=189-217) months, 95%) (Figure 3C) (Table IV).

For MSH6, positive staining was found in ten (15%) patients and negative staining in 57 (85%) patients. Patients with expression of MSH6 had a significantly (p=0.006) shorter survival time (80 (95% CI=36-125) months, 40%) than patients without expression of MSH6 (166 (95% CI=145-186) months, 5-year survival rate 74%) (Figure 4A, Table II). The survival times were significantly (p<0.0001) shorter for patients with expression of MSH6 and non-responders to chemotherapy (33 (95% CI=0-70) months, 20%) than for patients without expression of MSH6 and no metastasis (178 (95% CI=154-202) months, 86%) (Figure 4B), Table III). Patients with expression of MSH6 and

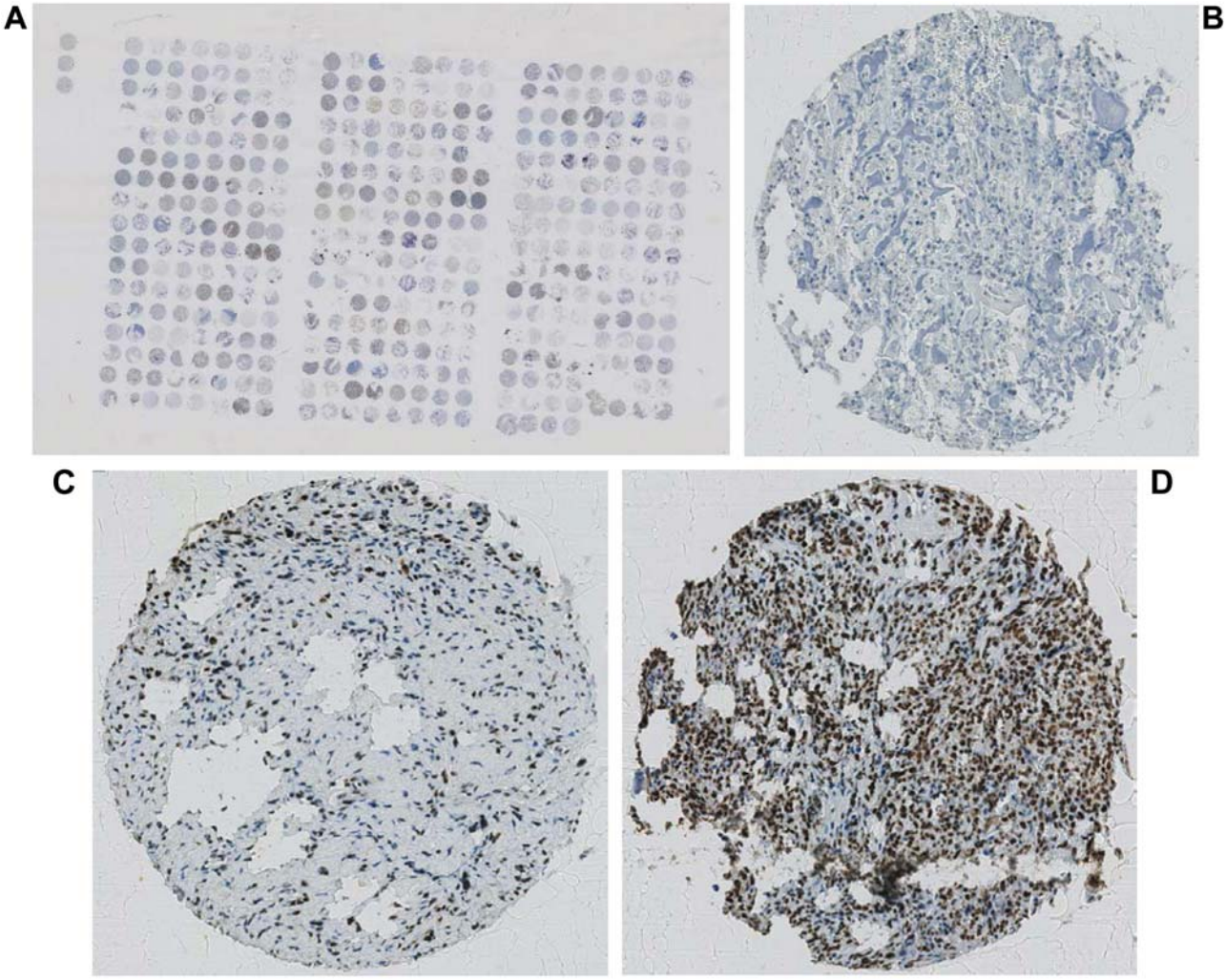

Figure 1. *MSH2 immunostaining on a tissue microarray slide is shown. Three vertical spots on the top left illustrate a control group, whereas the 404 spots on the right belong to surgical primary tumor resections of osteosarcoma patients (A). Lack of a brown reaction product is seen in negatively-graded spots of grade 1 (B). Brown nuclear staining in spots graded with score 2 (C). Nuclear expression in practically all tumor cells is seen in spots graded as score 3 (D).*

metastasis also had a significantly ($p<0.0001$) shorter survival time (47 (95% CI=9-85) months, 29%) compared to patients without expression of MSH6 and no metastasis (203 (95% CI=190-216) months, 95%) (Figure 4C) (Table IV).

When combining the staining results of MSH2 and MSH6 (MSH2/6), expression of MSH2/6 was observed in six (9%) patients and no expression in 54 (81%) patients. Expression of MSH2 and no expression of MSH6 were detected in three (5%) patients, while no expression of MSH2 and expression of MSH6 were identified in four (6%) patients. Patients with expression of MSH2/6 had significantly ($p=0.018$) shorter survival times than patients without expression of MSH2/6 (82 (95% CI 22-141) months and 50% compared to 163 (95% CI 142-185) months and 63%) (Figure 5A) (Table II). Furthermore, patients with expression of MSH2/6 and non-

Table II. *Patient survival and p-values (log-rank test) are shown for MSH2, MSH6 and MSH2/6.*

	Positive	Negative	*p*-Value
MSH2			
Patients	n=9	n=58	0.292
Survival time (months)	103±23	160±11 m	
5-year survival	67%	74%	
MSH6			
Patients	n=10	n=57	0.006
Survival time (months)	80 (36-125)	166 (145-186)	
5-year survival	40%	74%	
MSH2 and MSH6			0.018
Patients	n=6	n=54	
Survival time (months)	82 (22-141)	163 (142-185)	
5-year survival	50%	63%	

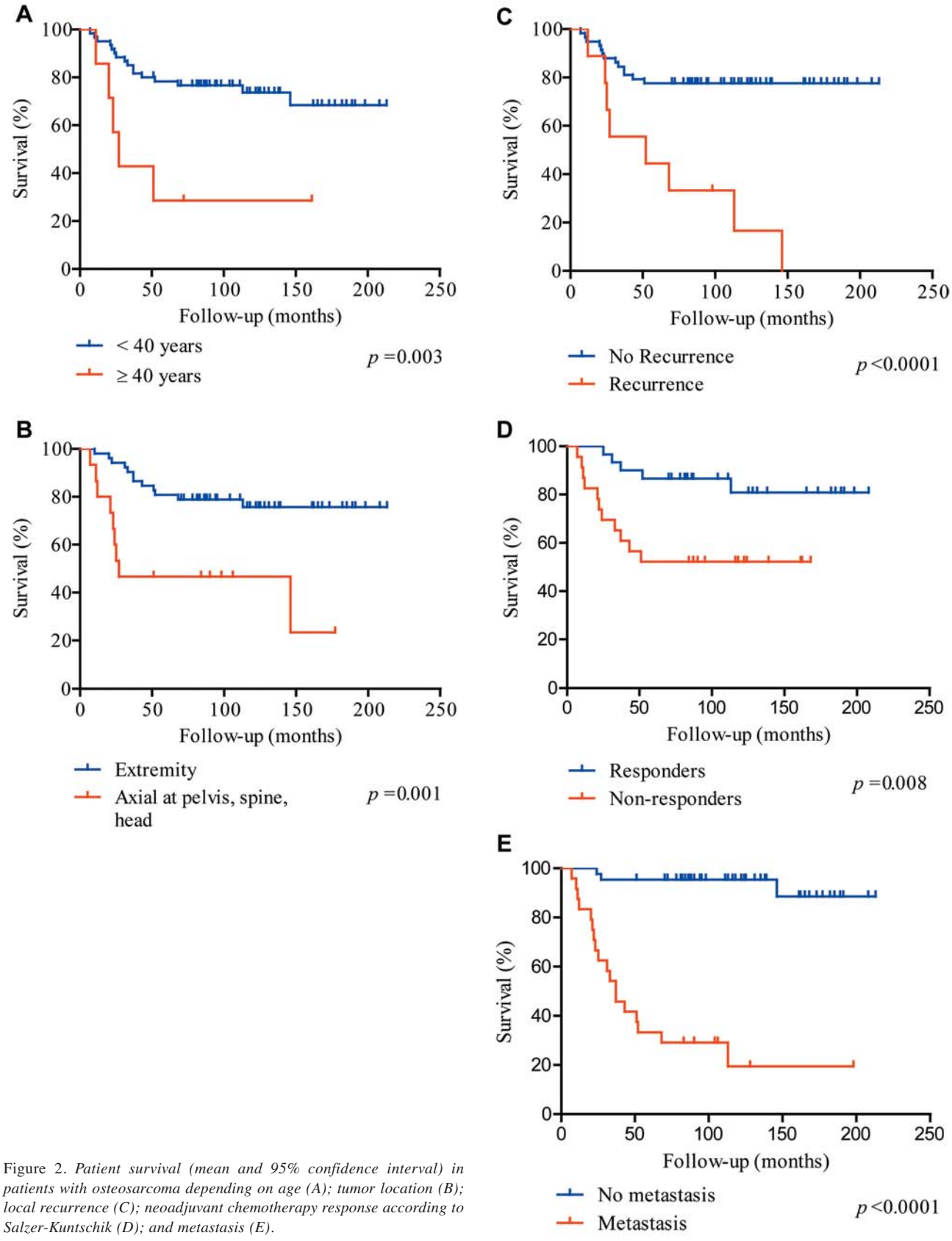

Figure 2. *Patient survival (mean and 95% confidence interval) in patients with osteosarcoma depending on age (A); tumor location (B); local recurrence (C); neoadjuvant chemotherapy response according to Salzer-Kuntschik (D); and metastasis (E).*

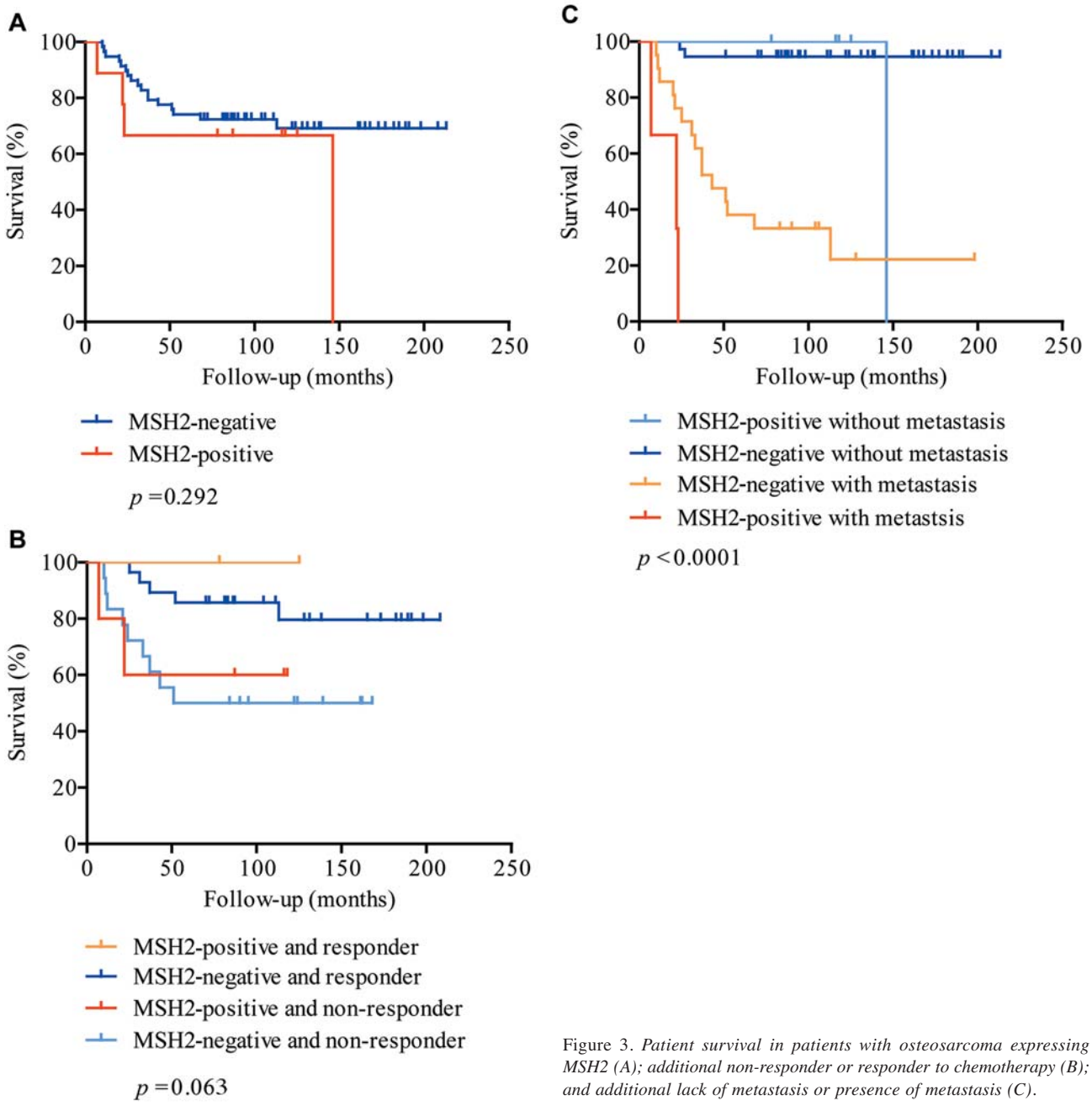

Figure 3. *Patient survival in patients with osteosarcoma expressing MSH2 (A); additional non-responder or responder to chemotherapy (B); and additional lack of metastasis or presence of metastasis (C).*

responders to chemotherapy had significantly (p<0.028) shorter survival times (48 (95% CI 0-102) months, 50%) than patients without expression of MSH2/6 and responders to chemotherapy (176 (95% CI 151-201) months, 85%) (Figure 5B) (Table III). Moreover, in patients with expression of MSH2/6 and metastasis (17 (95% CI=7-27) months, 0%), survival times were significantly (p<0.0001) shorter than in patients without expression of MSH2/6 and no metastasis (203 (95% CI=189-217) months, 94%, Figure 5C) (Table IV).

Discussion

The present study is the first to investigate the local expression of MSH2 and MSH6 as potential biomarkers in a series of OS patients. We showed that local expression of MSH6 and MSH2/6 in surgical primary OS tissue specimens was associated with significantly shorter survival times. This observation was especially pronounced in non-responders to chemotherapy and patients with metastases.

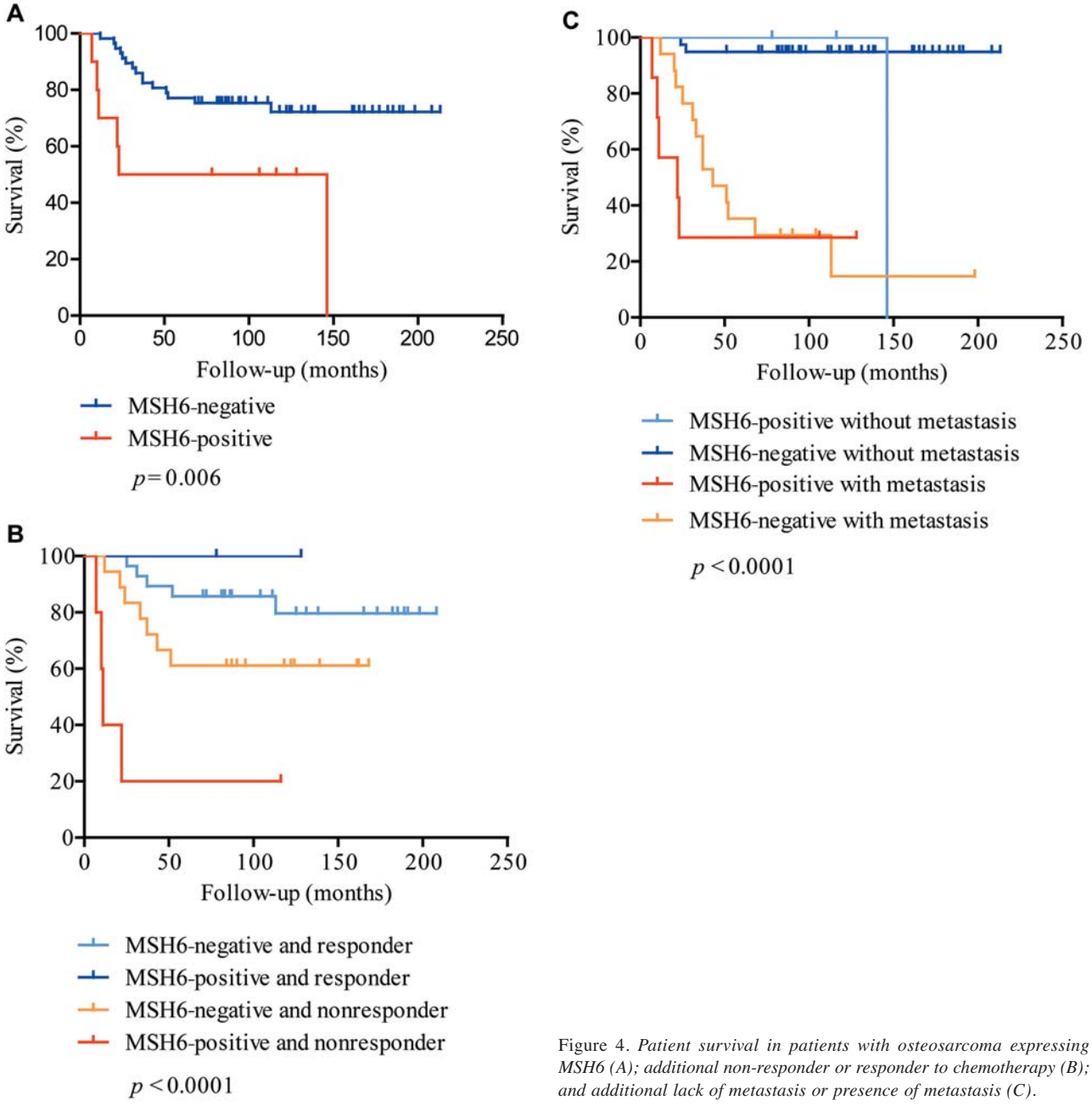

Figure 4. *Patient survival in patients with osteosarcoma expressing MSH6 (A); additional non-responder or responder to chemotherapy (B); and additional lack of metastasis or presence of metastasis (C).*

Our findings expand the knowledge about MSH2 and MSH6 and their roles in OS patients. Interestingly, alterations in the expression of MMR proteins, particularly MSH2, have recently been associated with OS formation for the first time in a case report by Ahmed *et al*. (29). However, this case report lacked a series of patients. Therefore, the present study adds valuable information to the current literature because it investigates the expression of MSH2 and MSH6 in a larger sample size and supports the importance of the MMR system in OS patients by showing that increased expression of MSH6 and MSH2/6 is associated with shorter survival times. Moreover, the present study suggests an even shorter survival time if expression of MSH6 and MSH2/6 is accompanied by non-response to chemotherapy (48) or metastasis (11).

Another study by Urso *et al*. (26) hypothesized that mutations in the *MSH2* and *MSH6* genes, which lead to a deficit in the respective proteins, are not only associated with colorectal cancer but also with sarcomas. In their

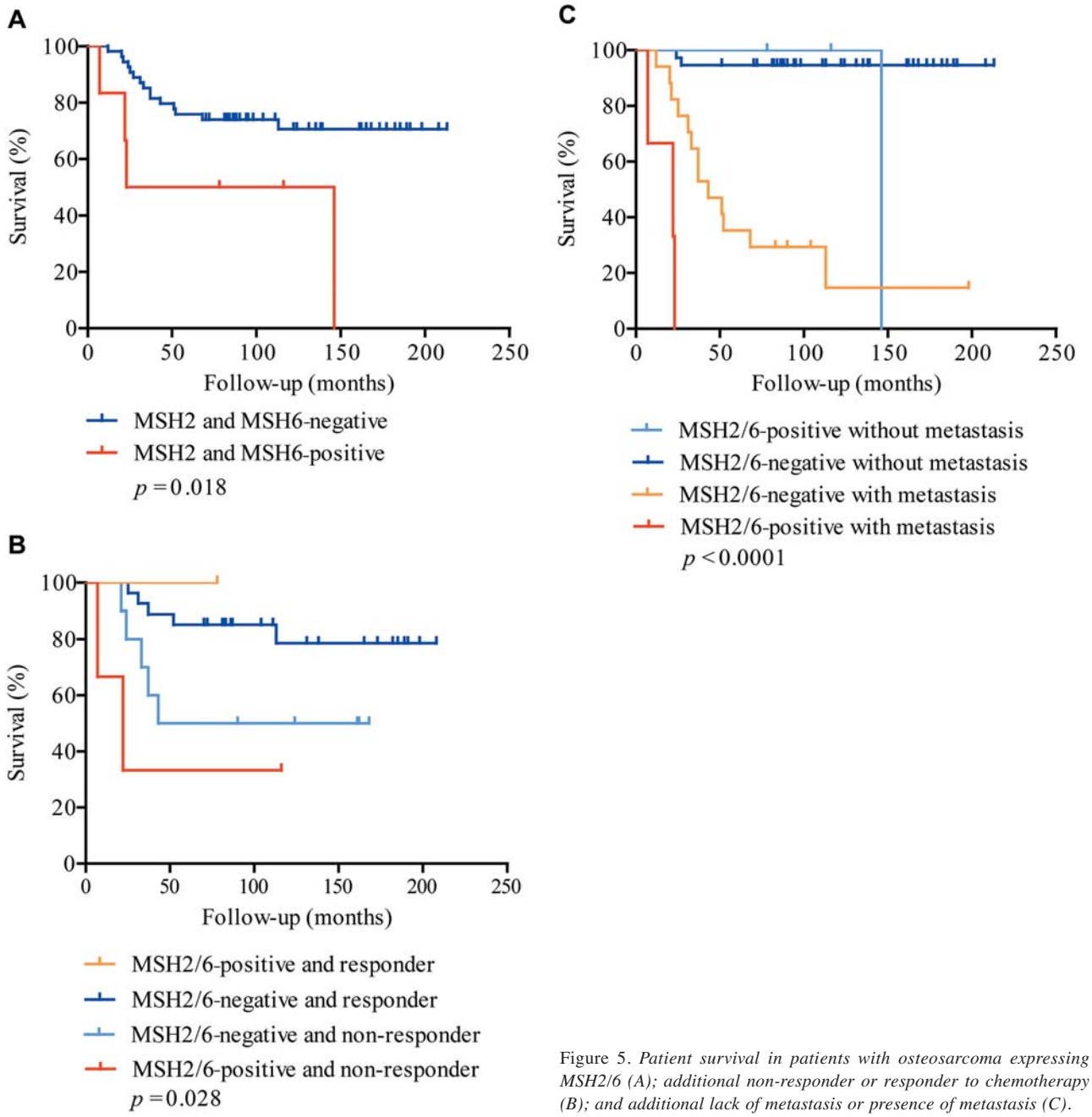

Figure 5. *Patient survival in patients with osteosarcoma expressing MSH2/6 (A); additional non-responder or responder to chemotherapy (B); and additional lack of metastasis or presence of metastasis (C).*

literature review, they found eleven cases of HNPCC (23, 24) patients that were coincidentally affected by sarcomas. Seven (63%) of those cases were affected by immunohistochemical loss of MSH2 and MSH6 expression. Furthermore, they presented a case with a genetic analysis of a leiomyosarcoma and detected deletions of exons on the MSH2 gene but not on the *MSH6* gene. This led to a defective MutSα because without its essential binding partner, MSH2, MSH6 could not be transported into the nucleus and, therefore, was not able to exert its MMR functions (32). While the findings of a decrease in MMR proteins by Urso *et al*. (26) may seem contradictory to our study at first sight, a more detailed analysis at their reported sarcoma types indicates no such contradiction. Interestingly, they were only able to include one patient with an actual OS from a study by Nilbert *et al*. (27) and this patient had neither a deficiency of MSH2 nor MSH6. Therefore, a lack of MSH2 and MSH6 seems to be

Table III. *Patient survival in patients with OS with regard to non-responders and responders to neoadjuvant chemotherapy, as well as expression of MSH2, MSH6 and MSH2/6.*

	Non-responders		Responders		*p*-Value
	Positive	Negative	Positive	Negative	
MSH2					
Patients	n=5	n=18	n=2	n=28	0.063
Survival time (months)	77 (32-121)	97 (65-130)	*	178 (154-202)	
5-year survival	60%	50%	100%	86%	
MSH6					
Patients	n=5	n=18	n=2	n=28	<0.0001
Survival time (months)	33 (0-70)	115 (84-146)	*	178 (154-202)	
5-year survival	20%	61%	100%	86%	
MSH2 and MSH6					
Patients	n=2	n=10	n=1	n=26	0.028
Survival time (months)	48 (0-103)	100 (57-142)	*	176 (151-201)	
5-year survival	50%	50%	100%	85%	

*No survival times were computed because all patients remained alive and were censored.

associated with various sarcomas other than OS. Importantly, the present study suggests that MSH6 and MSH2/6 play a crucial role in the aggressiveness of OS leading to shorter survival times.

A study by Fujii *et al*. (28) was able to show that increased expression of MSH2 may be associated with resistance to chemotherapeutic drugs. Apart from showing that stem-like OS cell lines possessed the ability to form sarcospheres and to self-renew, increased levels of DNA repair enzymes may have been associated with resistance to doxorubicin and cisplatin, which are both used in current chemotherapy protocols against OS. Another possible explanation may be found in a previous study by Belloni *et al*. (49), which demonstrated increased levels of MSH2 and MSH6 in differentiated neuroblastoma cells after treatment with doxorubicin. In contrast, undifferentiated neuroblastoma cells did not respond to doxorubicin treatment by increasing MSH2 and MSH6 protein levels. This study proved the involvement of MSH2 and MSH6 in a doxorubicin-related DNA damage response, which strongly depends on the differentiation state of the cells and post-transcriptional modifications of MSH2 and MSH6 mRNA (49).

Traditionally, the loss of immunohistochemical expression of MMR proteins has been shown to provide a selective advantage in tumorigenesis of tumor cells (50) by increased genomic mutation rates (51, 52), microsatellite instability and altered lengths of small tandem repeats (53), as well as loss of apoptosis (54). But a comprehensive review by Edelbrock *et al*. (30) revealed that there are many unsolved questions regarding the MMR system and that, in fact, interactions of MSH2 and MSH6 with other repair proteins, such as MutY homologue (MYH) (55) or the nucleotide excision repair (NER) machinery, may be responsible for more aggressive tumor growth. Increased expression of MMR proteins may lead to an increased repair capacity of interstrand crosslinks (ICL), which are typically formed by chemotherapeutic agents, such as cisplatinum (30). This could be attributed to the actions of MutSβ, a heterodimer of MSH2 and MSH3, which may be important for the incisional activity of ICL, as well as the nucleotide excision repair (NER) (56). Furthermore, the presence of MutSα may increase the resistance to cisplatinum through interaction with NER proteins; however, contrasting results have been found so far (30, 57, 58). When interpreting the results of our study, expression of MSH6 and MSH2/6 seems to increase chemoresistance of OSs and, in turn, progression of disease similar to what was shown for lung cancer (59). The pathomechanisms leading to over-expression of MMR proteins requires further investigations and future studies may address this interesting question.

The present study investigated the same patients and used a similar setup as in a recently published work by Jentzsch *et al*. (39). Apart from the investigation of two additional biomarkers, the present study also reports additional, valuable information about general patient demographics of the studied patients. Patients were reviewed retrospectively over a period of 18 years due to the relatively rare prevalence of OS. However, the characteristics of OS patients in our study are in line with results from a previous study by Bacci *et al*. (20) that showed shorter survival times for elderly patients, axial tumors at the spine or pelvis, local recurrences, non-responders to chemotherapy and metastasis.

An inherent limitation of a TMA is its limited power to quantify antigen expression. Nevertheless, advantages, such

Table IV. *Patient survival in OS patients with expression of MSH2, MSH6 and MSH2/6 as well as lack or presence of metastasis.*

	Metastasis		No metastasis		*p*-Value
	Positive	Negative	Positive	Negative	
MSH2					
Patients	n=3	n=21	n=5	n=37	<0.0001
Survival time (months)	17 (7-27)	79 (51-106)	146 (146)	203 (189-217)	
5-year survival	0%	29%	80%	95%	
MSH6					
Patients	n=7	n=17	n=3	n=39	<0.0001
Survival time (months)	47 (9-85)	71 (39-103)	146 (146)	203 (190-216)	
5-year survival	29%	24%	67%	95%	
MSH2 and MSH6					
Patients	n=3	n=17	n=3	n=37	<0.0001
Survival time (months)	17 (7-27)	71 (39-103)	146 (146)	203 (189-217)	
5-year survival	0%	24%	67%	94%	

as coverage of more than 90% of the tumor heterogeneity (45) or the simultaneous immunohistochemical evaluation of different tumor biomarkers in multiple patients (17, 19, 39, 42, 43), justify the use of a TMA. Another limitation of the present study is a relatively small sample number and the use of surgical primary tumor tissue resections after chemotherapy instead of biopsies before chemotherapy. However, these resections may be particularly suited to show over-expression of MMR proteins that may play an important role in chemotherapy resistance. Furthermore, current clinical practice shows that patient prognosis and decision on the use of adjuvant chemotherapy protocol is based on the extent of necrosis of tumor tissue after neoadjuvant chemotherapy and surgery. Therefore, local expression of MSH6 and MSH2/6 in surgical primary tumor resections may serve as valuable discriminative parameter for treatment decisions including adjuvant chemotherapy, prediction of survival times and education of patients.

Conclusion

The survival time of patients with OS may be negatively influenced by local expression of MSH6 and MSH2/6 in surgical primary tumor resections, which is particularly the case for non-responders to chemotherapy and patients with metastasis. These findings ask for an evaluation of the presented markers in a larger patient cohort in orden to study their potential role in OS progression. We recommend further molecular studies to clarify the involvement of MSH6 in OS patients during current standard chemotherapy.

Conflicts of Interest

The Authors declare that they have no competing interests.

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