A Tissue Microarray Study of Osteosarcoma: Histopathologic and Immunohistochemical Validation of Xenotransplanted Tumors as Preclinical Models

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Background: Osteosarcomas (OS) are aggressive neoplasms with a wide range of morphologic patterns.

Materials and Methods: OS cases (primary and xenotransplanted) with paraffin blocks available were collected and included in tissue microarrays (TMAs). A morphologic evaluation including the different passages in mice was carried out according to the new WHO criteria. In addition, TMAs were analyzed with a wide panel of immunohistochemical (IHC) markers (osteonectin, osteocalcin,cytokeratin, S100, Sox-9, Ki-67, Bcl-2, p53, p16, survivin, CD99, and caveolin-1).

Results: A total of 61 cases were collected. The distribution of the cases according to the histopathologic pattern was: 38 osteogenic OS, 8 primary chondrogenic OS, 2 primary telangiectatic OS, 6 parosteal OS, 2 primary small cell OS, 2 primary poorly differentiated OS, 1 primary dedifferentiated OS, and 3 primary pleomorphic MFH-like OS. The tumor morphology in xenotransplants was similar to the primary or metastatic tumor of origin and was generally maintained over the passages. The IHC results were heterogeneous and osteonectin and osteocalcin were the most expressed in original tumor and xenografts. S100 and Sox-9 were expressed in chondrogenic areas. Caveolin and survivin showed significant IHC variation between the subsequent passages. p16 displayed heterogenic expression. p53 expression increased over the passages, and Ki-67 expression was not associated with a more undifferentiated pattern, but increased over the passages.

Conclusions: An accurate morphologic evaluation using TMAs in original tumor is essential for the OS diagnosis; hence there is no IHC marker that alone distinguishes the OS subtypes. Xenografts in OS allow the study of tumor progression in this type of aggressive neoplasm.

Key Words: osteosarcomas, immunohistochemical, TMAs, xenografts

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Osteosarcoma (OS) is a malignant primary bone tumor that commonly affects adolescents and young adults.[1] The evolution and prognosis are poor and the treatment currently includes neoadjuvant chemotherapy and conservative surgery.[1–3] With regard to location, intramedullary OS is the most frequent, although parosteal, multifocal, intracortical, and surface OS have also been described.[1,4] OSs are aggressive neoplasms with a wide range of morphologic patterns. Their accurate diagnosis improves treatment and patient survival.[5] The most frequent histologic subtypes include osteoblastic, chondroblastic, and fibroblastic OS, although infrequent subtypes such as telangiectatic and small cell/microcellular OS have also been reported.[6,7] Immunohistochemical investigation is not generally useful for diagnosis, but can be very helpful for accurate diagnosis in undifferentiated tumors and small round cell tumors arising in the bone.[1,7] Osteonectin and osteocalcin have been used for diagnostic purposes, being specific for osteogenic tumors and highly expressed in OS.[8–10] Cytogenetic studies have revealed heterogeneity related to karyotypic complexity, with polyploid karyotype with several numeric chromosomal alterations.[11–13] Abnormalities in p16 and p53 have been reported.[14–18] An alteration of p53 may predict a poor outcome in OS patients, and the loss of p16 expression correlates with decreased survival in pediatric OS. Caveolin-1 expression has been related to a better outcome with no metastatic potential of OS.[19]

Usually, bone tumor biopsies are very small, especially with the use of needle biopsies, with scant material being left over for extra studies after diagnostic purposes. Xenograft models of bone tumors are of great value,[20] not only because xenotransplanted tumors offer an easy source of fresh material, but they are also a resource for in vivo experiments. Furthermore, this model allows the characterization of a large number of tumors using histopathology, immunohistochemistry (IHC), ultrastructural microscopy, cytogenetics (conventional and fluorescence

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in situ hybridization/FISH), and molecular biology, and investigations of tumor biology in nude mice. OS xenografts can also be generated from OS cell lines and can be very important in the search for tumor progression, metastatic potential, angiogenic capacity, and response to therapy.[21,22]

Tissue microarray (TMA) technology allows the assessment of histopathology, IHC, and molecular alterations in different tumors from a large cohort of patients on a single slide.[23–25] In addition, TMAs allow the examination of serial sections obtained from the same tumor specimen and xenotransplanted tumor.[26] Currently, the emerging use of TMAs leads to savings in cost, time, and fundamentally, tissue. Therefore, several laboratories apply the use of TMAs to archival samples to save room for the storage of paraffin blocks. Few TMA studies other than mainly therapeutic proposals have been reported in OS.[27]

Xenograft models of OS, including successive generations of passages in mice, in combination with TMA technology have not been reported so far. Furthermore, TMAs of a large cohort of OS cases with respect to histopathology and IHC have not yet been reported. Therefore, this study aims to make a morphologic and immunohistochemical analysis of a large and heterogeneous group of OS by means of TMAs, with their successive passages after xenotransplantation into nude mice. In a separate study, we investigate the representativity of xenotransplanted OS tumors by the use of genomic profiling (Kresse et al, unpublished).

MATERIAL AND METHODS

Case Selection/Sample Sources

We collected cases with diagnosis of OS (primary tumors, recurrences and/or metastasis, and xenotransplanted OS) with paraffin block available from the files of the Department of Pathology, University of Valencia, Spain; and from the Departments of Pathology and Tumor Biology, The Norwegian Radium Hospital, Oslo University Hospital, Norway. Considering the fact that some of the cases were archival cases, where necessary these cases were reviewed and reclassified according to the new WHO criteria[1] for OS diagnosis in hematoxylin-eosin (H/E) stained slices.

Assembly of TMAs

Tissue microarraying was carried out using a manual tissue arrayer (Beecher Instruments, Sun Prairie, WI). Two cores (1 mm in thickness) of each sample were included, except for the cases with a heterogenic morphologic pattern, from which more than 2 cores were included. The cases were distributed into 3 groups: (A) primary/recurrence and/or metastatic tumors; (B) primary/recurrence and/or metastatic tumors and their corresponding nude mice xenografted material; (C) xenografted OS with no original tumor block available. Owing to the wide variability in number of passages among the xenografted cases (between 1 and 68), we selected different passages of each case depending on the quality of the original block and the total number of passages. For presentation of the results and the statistical analysis of the data, the cores from the passages were grouped into: initial passages,[1–4] middle passages,[5–10] and late passages (≥ 11). In every TMA, 2 cores of normal liver tissue were included as a control.

Thirteen TMAs were constructed, distributed as: (a) 2 TMAs from 22 original cases with 22 samples in 44 cores, (b) 8 TMAs from 31 original cases with xenografted material with 184 samples in 368 cores, and (c) 3 TMAs from 8 xenografted cases with no original tumor available with 57 samples in 114 cores.

After TMA construction, an H/E stained section of each TMA was carried out; first to confirm the presence of an intact and representative neoplasm and second to analyze the morphology, not only in the original cases, but also throughout the passages in nude mice. In addition, 5-μm sections were cut to carry out IHC staining.

Immunohistochemical Analysis

IHC analysis was done using antiosteonectin antibody (Novocastra) at 1:50 dilution, antiosteocalcin antibody (Biogenesis) at 1:250 dilution, anti-CD99 antibody (clone 12E7, DakoCytomation) at 1:50 dilution, anti-Sox-9 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100 dilution, anti-S100 polyclonal antibody (DakoCytomation) at 1:200 dilution, antisurvivin polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:50 dilution, anti-p16 antibody (clone F12, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100 dilution, anti-p53 antibody (clone DO7, Novocastra) at 1:50 dilution, pan-cytokeratin (CK) (AE1/AE3) antibody (DakoCytomation) at 1:50 dilution, anti-Ki-67 antibody (MIB-1, DakoCytomation) at 1:50 dilution, anticaveolin (CAV) polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200 dilution and anti-Bcl-2 antibody (clone 124, Novocastra) at 1:50 dilution. Antigen retrieval was carried out by pressure cooker boiling for 3 minutes in 10 mmol/L of citrate buffer (pH 6.0). The LSAB method (DakoCytomation) was used, followed by revelation with 3,3′-diaminobenzidine. Cytoplasmic and/or membrane staining was considered positive for osteonectin, osteocalcin, CD99, CK, caveolin, S100, and Bcl-2 antibodies, and nuclear staining was considered positive for Sox-9, survivin, p53, p16, and Ki-67 antibodies. Sections were examined and immunoreactivity was defined considering staining intensity and percentage of positive cells as: negative: fewer than 5% of tumor cells stained; low positivity (+): 5% to 10% of tumor cells stained; moderate positivity (++): 10% to 50% of tumor cells stained and strong positivity (+++): more than 50% of the tumor cells were stained. All sections were evaluated blindly by 3 pathologists (EM, IM, and ALLB). Staining intensity agreement was recorded, and in cases of disagreement, the score was determined by consensus.

Xenograft Model

Male nude mice were purchased from IFFA-CREDO (Lyon, France) and kept under specific pathogen-free

conditions throughout the experiment (with vinyl isolates plus sterilized food, water, cage, and bedding). The specimens for xenografting were obtained from the surgery of original tumors and placed in culture medium (RPMI 1640) with antibiotic at 37°C until transplantation (usually less than 2 h after surgery). Various fragments of nonnecrotic tumor, about 3 to 5 mm in size, were xenografted into the subcutaneous tissue of the backs of 2 nude mice. After allowing growth, the subsequent tumor transfers were carried out after the same procedures as in the initial xenotransplant and always under highly sterile conditions. In each passage, sufficient material was obtained for histopathology analysis (FFPE blocks from which TMAs were constructed), touch preparations, electron microscopy, tissue culture, and frozen tissue. All experimentation involving laboratory animals was approved by the Institutional Animal Care of Valencia University and Local Government and carried out in accordance with national legislation.

Statistical Analysis

The statistical analysis was done with SPSS software version 15.0. Fisher test was used to compare proportions as appropriate in each case.

RESULTS

Sixty-one OS with paraffin blocks available, 45 cases from Valencia and 16 from Oslo, were collected. The distribution of the cases according to their histopathologic pattern and origin were: 38 osteogenic OS (27 primary, 5 metastatic and 6 primary and metastatic), 8 chondrogenic OS (7 primary and 1 metastatic), 2 telangiectatic OS (1 primary and 1 metastatic), 6 parosteal OS (5 primary and 1 metastatic), 2 primary small cell/microcellular OS, 2 primary poorly differentiated OS, 1 primary dedifferentiated OS, and 3 primary pleomorphic MFH-like OS.

The osteoblastic cases consisted of atypical cellularity admixed with an intercellular pink, dense, and irregular osteoid. The tumor cells were, depending on the case, fusiform, ovoid, large cells, epithelioid, or a mixture of these. The most frequent growth patterns were solid, diffuse, angiocentric and somewhat cord-like, and secondary, hemangiopericytoid, and storiform. The original morphologic pattern was maintained in their metastases; with an increasing number of tumor cells, mitotic, and apoptotic figures. Four osteoblastic cases also showed an associated secondary pattern, consisting of fibroblastic areas (EOS 68), telangiectatic areas (EOS 19 and 69), and pleomorphic areas (EOS 72), but without constituting sufficient criteria for a different diagnosis.

In the majority of the xenografted cases, 21 of 31 from group B, the histopathology pattern was similar to the primary or the metastatic tumor, also with some features of a higher aggressivity, such as increased cellularity, mitotic and apoptotic figures, and necrotic areas. However, in 10 xenografted cases, the morphology changed over the passages and was quite different from the original tumor implanted into nude mice. Phenomena such as dedifferentiation (EOS 61, 67, 71, and 72), differentiation (with osteoid formation) (EOS 65), and acquisition of new patterns were observed. These new patterns consist of giant cell (EOS 17 and 64), microcellular (EOS 68 and 69), and chondroid (EOS 63) (Fig. 1).

IHC results were heterogenous (Tables 1, 2), although some associations were found in the statistical analysis (Tables 3, 4). Osteonectin and osteocalcin were the most-expressed markers, being retained, despite the change in the morphology during the subsequent passages and even when an undifferentiated morphology was present (Fig. 2). S100 and Sox-9 were expressed mostly in chondrogenic cases; however, no statistical differences related to Sox-9 were found in nonchondrogenic OS. Six of the 8 chondrogenic cases showed nuclear, moderate, or high Sox-9 positivity, whereas 10 osteogenic, 1 telangiectatic, 1 dedifferentiated, and 1 pleomorphic OS also showed positivity. However, this was mainly low, and in 1 case, moderate with abundant associated cytoplasmic expression. CK was positive in only 4 original osteoblastic OS, in which its positivity increased over the passages, especially in the later ones. Bcl-2 was positive only in 2 original osteoblastic OS, and revealed generally low and heterogeneous expression throughout the mice passages with a statistically significant higher expression in the late passages. CD99 was positive in 1 of the 2 microcellular cases with a marked cytoplasmic expression. Nevertheless, this expression was not exclusive to this OS variant; other cases also showed positivity. Caveolin and survivin showed immunoreactivity in the majority of the tumors, with no significant variation among the subtypes, except with expression of the former in 12 out of the 14 metastatic cases. Survivin was significantly higher over the subsequent passages. p16 displayed heterogenic expression with no significant differences among the OS subtypes or the subsequent passages. However, 19% of all the OS lost their expression. p53 expression increased over the passages, although the statistical analysis was not significant. The proliferation index (Ki-67) was not associated with a more undifferentiated histopathology pattern of the tumor, but increased significantly over the passages.

DISCUSSION

New ancillary technologies such as TMAs are very helpful for the diagnosis and systematic study of a large cohort of tumors. Packeisen et al[23] reviewed in detail the use of this high-throughput source in daily diagnosis and research, drawing attention to the central position of pathology in the evolution of cancer investigation. Nilbert et al[28] applied this technology to sarcomas and compared its use with the conventional paraffin blocks, finding the most limiting feature of TMAs to be the lack of representation of the whole tumor. In our opinion, an accurate study of the original case to choose a representative area, together with the inclusion of more than 1 core and care in assembling the TMA, is a requirement for the reliability of the TMA.

Evaluating a large cohort of OS in 1 slide is of great value, especially in series such as ours, in which many

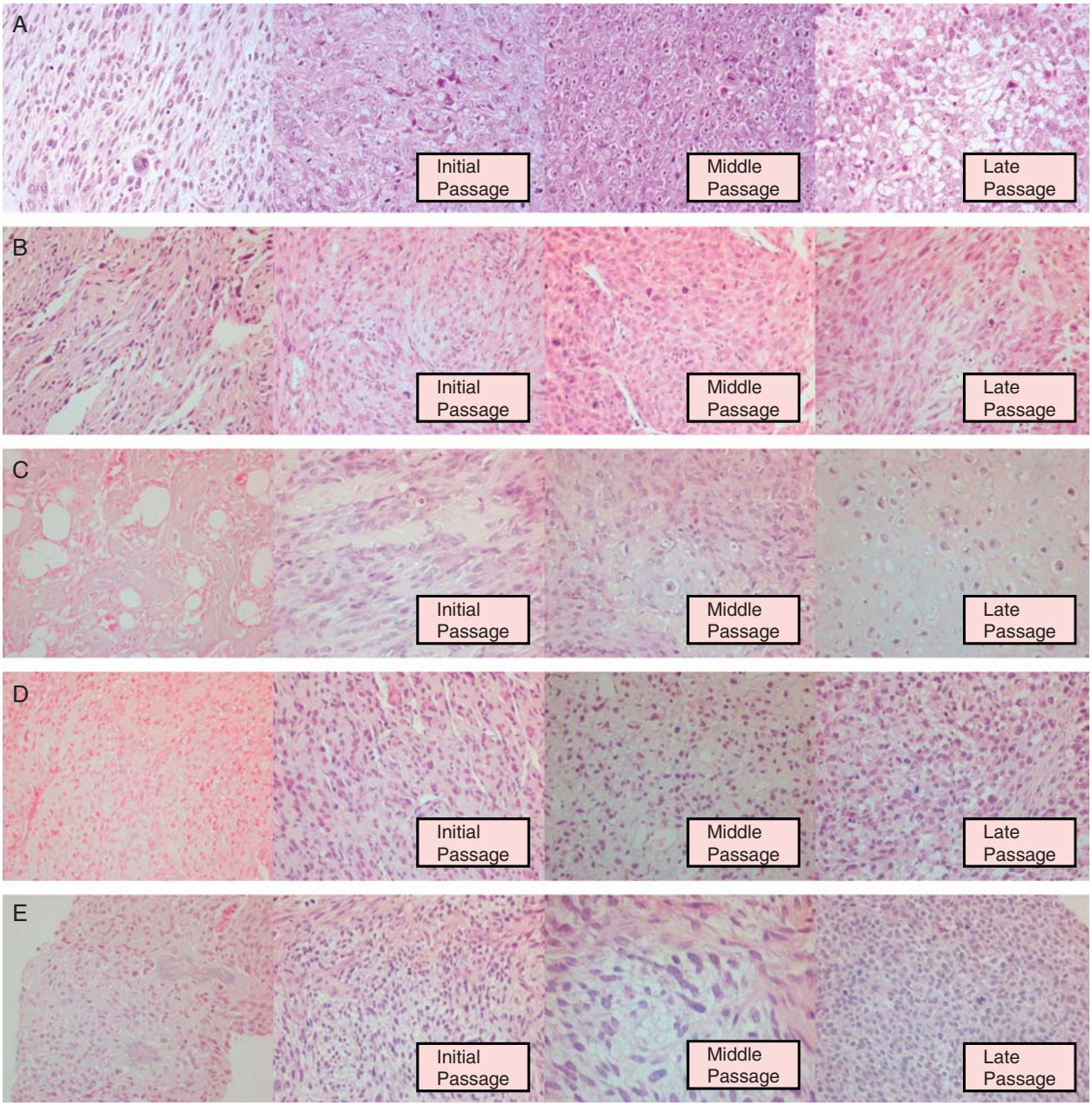

FIGURE 1. Osteosarcoma (OS) cases with morphology changes during the nude mice passages. A, EOS17 An osteoblastic OS that changes into a Giant cell OS. B, EOS65 Differentiation with osteoid formation. C, EOS63 A parosteal OS that shows chondroid change. D, EOS61 Dedifferentiation. E, EOS68 Dedifferentiation to a small cell osteosarcoma.

different OS subtypes are present, including unusual variants. Hauben et al[5] showed the importance of classifying high-grade OS, not only for predicting the response to treatment, but also for the overall survival of the patients. Therefore, 13 TMAs with 61 OS cases are now available for testing new antibodies and for carrying out genetic studies that could improve the treatment and diagnosis, and opening up new approaches to OS. In addition, constructing TMAs from xenografted material improves the knowledge related to the evolution of the histopathologic pattern and immunohistochemical expression of the OS over subsequent passages; as has been proven in synovial sarcomas and chondrosarcomas by Subramaniam et al and Machado et al, respectively.[24,26]

The construction of the TMAs from the xenograft tumors included cores from initial (passage 1 to 4), middle (5 to 10), and late ($\geq$ 11) passages. The wide experience of our group working for over 20 years with the xenograft platform,[20] allows us to divide them into these groups based upon earlier experience.[26] The tumor characteristics become established after the fourth passage, and with this distribution of passages, the morphology and behaviour

TABLE 1. Immunoprofile in Each Original Osteosarcoma Subtype

OS subtype	OS case	Surv	Sox-9	Cav	Osn	Osc	p16	S100	CD99	CK	Ki-67	p53	Bcl-2
Osteoblastic	3												
	11												
	12												
	13												
	13*												
	14												
	17												
	19												
	19*												
	21*												
	28												
	31												
	34												
	34*												
	42												
	44												
	44*												
	45p												
	45*												
	48												
	48*												
	49												
	50												
	51												
	52												
	54												
	55*												
	58												
	61												
	64												
	66												
	68												
	69												
	70												
	72												
Chondrogenic	16												
	41												
	59												
	35												
	47												
	60												
	71												
Parostal	5												
	10												
	36												
	37												
	63												
Telangiectatic	57												
	67												
Microcellular	7												
	24												
Pleomorphic	6												
	9												
	65												
Poorly dif.	43												
Dedifferentiated	62												

Positive cases in green, negative cases in red, and noninformative cases in white.
*Metastatic cases.

TABLE 2. OS Cases in Which the Morphology Changes Over the Nude Mice Passages

EOS	Histopathology in TMA	Origin	Psg	Surv	Sox-9	Cav	Osn	Osc	p16	S100	CD99	CK	Ki-67	p53	Bcl-2
17	Osteogenic OS	Primary													
17	Large cell OS	Nude	Initial												
17	Large cell OS	Nude	Middle												
17	Large cell OS	Nude	Late												
61	Osteoblastic OS	Primary													
61	Osteoblastic OS	Nude	Initial												
61	Dedifferentiated OS	Nude	Middle												
61	Dedifferentiated OS	Nude	Late												
64	Osteoblastic OS	Primary													
64	Osteoblastic OS	Nude	Initial												
64	OS with prominent giant cells	Nude	Middle												
64	OS with prominent giant cells	Nude	Late												
67	Osteoblastic OS	Primary													
67	Osteoblastic OS	Nude	Initial												
67	Osteoblastic OS	Nude	Middle												
67	Undifferentiated tumor with osteogenesis	Nude	Late												
68	Osteoblastic OS	Primary													
68	Osteoblastic OS myxoid areas	Nude	Initial												
68	Small cell round undifferentiated tumor	Nude	Middle												
68	Small cell round undifferentiated tumor	Nude	Late												
69	OS with prominent giant cells	Primary													
69	OS with prominent giant cells	Nude	Initial												
69	OS with prominent small round cells	Nude	Middle												
69	OS with prominent small round cells	Nude	Late												
72	Osteoblastic OS	Primary													
72	Pleomorphic OS with spindles cells	Nude	Middle												
72	Pleomorphic OS with spindles cells	Nude	Late												
71	Chondroblastic OS	Primary													
71	Chondroblastic OS	Nude	Initial												
71	Chondroblastic OS	Nude	Middle												
71	Pleomorphic OS	Nude	Late												
63	Parosteal OS	Primary													
63	Parosteal osteogenic OS	Nude	Initial												
63	Osteogenic OS	Nude	Middle												
63	OS with chondroblastic areas	Nude	Late												
65	Pleomorphic sarcoma (MFH-like OS)	Primary													
65	Pleomorphic sarcoma (MFH-like OS)	Nude	Initial												
65	Pleomorphic sarcoma (MFH-like OS)	Nude	Middle												
65	MFH-like OS with osteoblastic	Nude	Late												

Marker expression is indicated with different green tonalities for positive cases from light (low expression) to dark (high expression) and red for negative cases. In white the noninformative cases.

of the tumors are comparable; not only among passages of the same group, but also with other xenografted tumors.

In our series, conventional OS were the most frequent, with the osteogenic as the predominant variant followed by the chondrogenic, as described in the literature.[1] No fibroblastic OS (the third in frequency) was included in this study; however 1 of the cases (EOS 68) showed an admixed pattern with osteoid and fibroblastic areas. A different type of matrix was also observed after the nude mice passages in a parosteal OS (EOS 63), which changed into a chondrogenic tumor, and a pleomorphic MFH–like OS (EOS 65), which changed into an osteogenic. Such findings have not earlier been reported and need to be contrasted with new cases to provide a better

TABLE 3. Comparison of the High Expression of the Markers Between Initial and Middle-late Passages in the Nude Mice

Immunomarker	Initial Passages (%)	Late Passages (%)	*P*
Ki-67	n = 3 (9.4)	n = 8 (32)	$P < 0.05$
p53	n = 3 (9.4)	n = 6 (24)	$P > 0.05$
p16	n = 5 (16.7)	n = 3 (13)	$P > 0.05$
Survivin	n = 16 (51.6)	n = 21(87.5)	$P < 0.05$
Caveolin	n = 25 (80.6)	n = 19 (79.2)	$P > 0.05$
Bcl-2	n = 3 (9.4)	n = 5 (20)	$P > 0.05$

p16 loss of expression (negativity) is compared between initial and middle-late passages.

TABLE 4. Comparison of Marker Expression Between Chondrogenic and Nonchondrogenic OS in Original Tumors

Immunomarker	Chondrogenic OS (%)	Nonchondrogenic OS (%)	*P*
Sox-9	n = 6 (75)	n = 13 (24.5)	$P > 0.05$
S100	n = 8 (100)	n = 28 (52.8)	$P < 0.05$

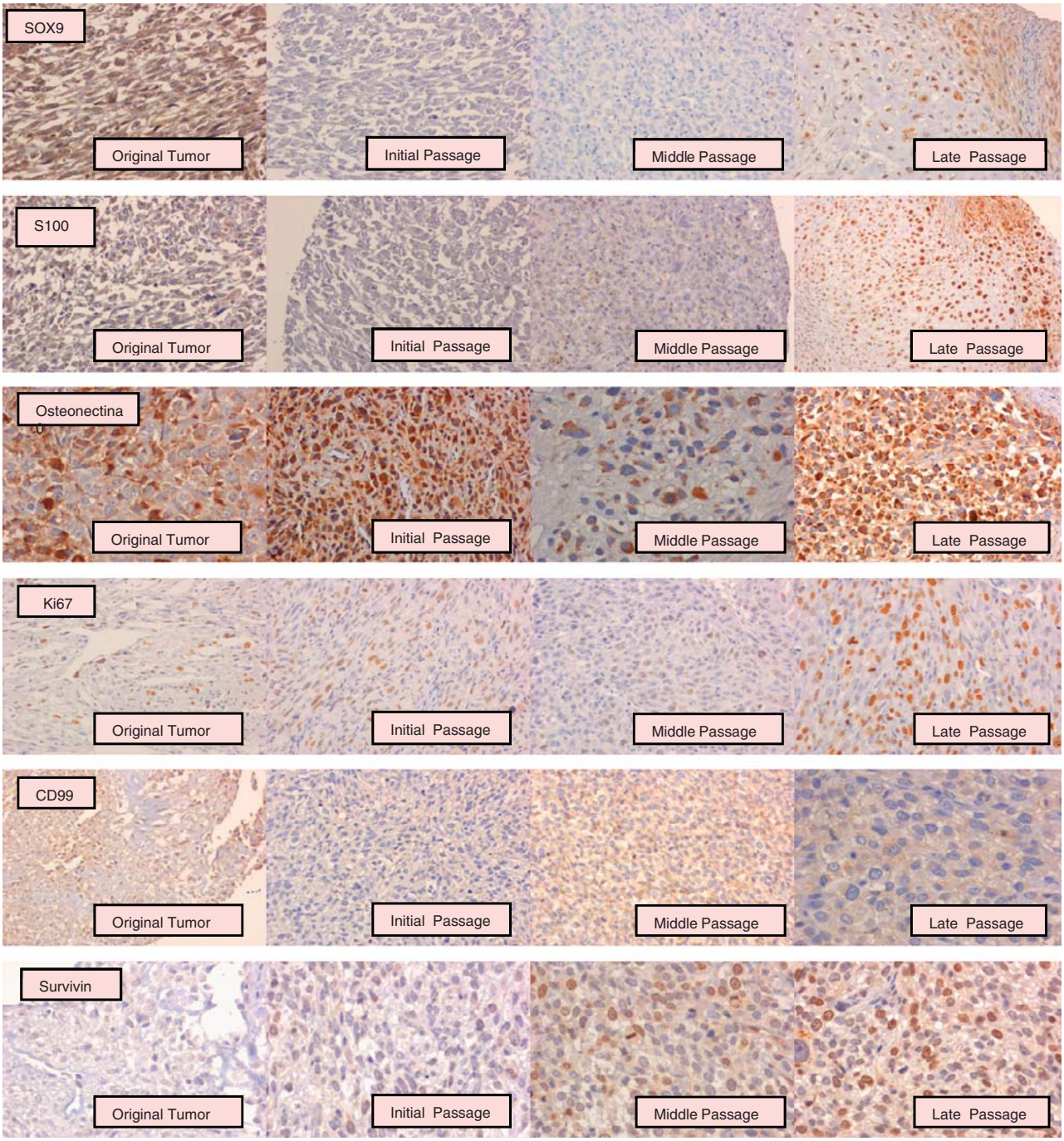

FIGURE 2. Immunohistochemistry in Osteosarcomas (OS) tumors and during the subsequent passages. SOX 9 and S100 expression in a Parosteal OS (EOS 63) in which the morphology changed into a chondrogenic tumor. Maintenance of the Osteonectin expression in an Osteoblastic OS (EOS 61), despite the change into an undifferentiated tumor. Ki 67 in a pleomorphic OS (EOS 65) which changed into an osteogenic tumor. CD99 and survivin expression in an Osteoblastic OS (EOS 68) in which the morphology changed into a microcellular tumor.

understanding. However, the changes in the matrix after xenografting, especially regarding bone formation, need to be confirmed as changes in the tumor cells and not being owing to the mouse stroma. Tokunaga et al[29] and Hara et al[30] reported that the mouse cells were induced to change into osteocytes by the inoculation of human OS cells, and were consequently responsible for bone formation. Good characterization of the origin of the cells, both morphologic and cytogenetically, using for instance cell cultures and FISH, will give clues to excluding this possibility.

Our study includes rare OS variants, 2 telangiectatic and 2 small cell OS, which account for 3% of all the OS, whereas the estimated incidence is 4% for telangiectatic OS and 1.5% for small cell OS.[1] We lack immunophenotypic markers for these 2 OS variants, although being aware that this type of OS exists and is especially important to exclude other bone tumors. Our series also included 2 extra cases (EOS 19 and 69) that showed large, but sporadic, blood lacunae surrounded by giant multinucleated cells; however, the complete case could unfortunately not be evaluated. Hence, these secondary patterns need to be better evaluated and understood to determine whether they are new potential subtypes or simply morphologic changes with no clinical meaning.

The 2 microcellular OS were compounds of a small, admixed cell population with scant and immature osteoid, as described by Nakajima et al.[31] According to the literature,[7,32] their immunohistochemical profile alone can be a pitfall when excluding other small cell tumors, especially when osteonectin or osteocalcin negative. In our cases, 1 was positive for osteonectin (EOS 7) and 1 for osteocalcin (EOS 24).

Regarding the IHC analysis, osteonectin and osteocalcin were generally expressed in all cases and subtypes, even in undifferentiated OS; these markers, especially osteonectin, are useful not only for the diagnosis of OS, but also for its differential diagnosis from other malignant bone tumors and to asses the origin of any metastasis. Positivity for these markers is not necessary to diagnose a tumor as OS, but if an undifferentiated tumor or a metastasis is positive for any of these 2 markers, it is highly suspicious of an OS.[8,10] However, Chano et al[9] observed a decrease in the osteocalcin expression in the metastasis when looking for the OS histogenesis, whereas in this study all the metastatic cases were positive.

OS are usually negative for Sox-9, except for the chondrogenic OS, in which some expression in chondrogenic areas is observed. However, in our series, although other OS variants showed positivity, the apparent expression was both nuclear and cytoplasmic, which leads us to doubt the reliability of the expression.

OS may express S100, being observed by Devaney et al in small cell OS[7] and Hasegawa et al[33] in chondroblastic, osteoblastic, low-grade central, giant cell-rich, and epithelioid OS subtypes. S100 expression in our study was significantly higher in the chondrogenic OS than in nonchondrogenic; although some expression was found in 50% of the osteoblastic, 80% of the parosteal, and in all the dedifferentiated and poorly differentiated OS.

OS may show positivity for CK,[34] although in our cases only 4 original osteoblastic OS expressed this marker. However, its positivity increased over the mice passages, especially in the later ones. This finding needs to be better understood, investigating for example other types of cytokeratins alone or in combination with the epithelial membrane antigen; as in already published studies.[35] Bcl-2 revealed generally low and heterogeneous expression with a statistically significant higher expression in the late passages in nude mice. CD99 was positive in every OS variant, including 50% of the microcellular cases. Despite being unspecific, this result must be taken into consideration, especially in the differential diagnosis of this entity. However, the expression was more cytoplasmic than membranous, as typically occurs in the Ewing family tumors.

Caveolin showed immunoreactivity in the majority of the tumors with no significant variation among the subtypes or subsequent passages; even in the majority of the metastatic cases. Nevertheless, with only 14 metastatic cases and without clinical data we cannot conclude that caveolin is a marker either for good or for bad prognosis, as already published by Cantiani et al.[19]

Survivin showed immunoreactivity in the majority of the tumors with no significant variation among the subtypes. However, surprisingly few of the tumors giving rise to xenografts were positive (20%), whereas the xenografts all became positive, which highlights the importance of this antiapoptotic protein in OS progression. Trieb et al[36] also studied survivin expression in OS and related its nuclear expression to a prolonged survival; however Osaka et al[37] detected survivin mRNA expression by RT-PCR in 22 OS with a significantly higher expression in metastatic cases and related to a poor prognosis, more in line with these observations. Despite being contradictory findings, both focus on the importance of this antiapoptotic pathway of progression and open up the possibility of its use as a pretherapy response predictor.

p16 and p53 displayed heterogenic expression with no significant differences between passages or histologic subtypes. However, 20% of our OS lost their p16 expression, similar to 17% of the cases of Maitra et al[14]; whereas for Benassi et al,[15] loss of p16 expression was found in 38% of the cases. Considering that 16% of our cases were inconclusive, with nuclear and extensive cytoplasmic overexpression, the percentage could probably be higher. p53 expression was generally low, except in the more undifferentiated cases and the late passages; a result that needs to be clarified because p53 mutation may play a role in neoplastic transformation[33] and probably in the evolution of OS.[17]

The proliferation index (Ki-67) was not necessarily associated with a more undifferentiated histology of the tumor, but increased significantly over the passages. These results agree with the results published by Jong et al,[38] in which the proliferative index did not seem to predict either disease-free or overall survival.

Extensive tissue sampling and an accurate morphologic evaluation of the tumors are essential for OS diagnosis; hence there is no single immunohistochemical marker that distinguishes the subtypes. However, osteocalcin and osteonectin are reliable markers for the diagnosis of OS, and the chondrogenic variant shows a significantly higher S100 expression than the others. Survivin and p53 expression increases with tumor evolution over the passages, and Ki-67 expression is related to tumor progression over passages.

On the basis of the detailed immunohistochemical analysis of the different xenograft passages, we must

conclude that, although not perfect, the xenograft models pretty well represent their tumor of origin. Obviously, phenotypes relying on stroma markers, or markers induced in cancer cells as a response to stroma signals, will not be represented well in such models. However, our parallel study of genomic characteristics of such xenografts (Kresse et al, unpublished) supports the overall good representation of human tumors by OS xenografts.

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