Immunoeexpression of Ki67, p53 and cyclin D1 in osteosarcomas

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Abstract

The main malignant tumor of the bone tissue is represented by osteosarcoma, neoplasia with a reserved prognosis and an unpredictable evolution, often aggressive. Cell cycle disruption is one of the complex biomolecular mechanisms involved in the progression of osteosarcomas. In this study, we analyzed the immunoeexpression of Ki67, p53 and cyclin D1 for 18 primitive osteosarcomas in relation to the clinicopathological prognosis parameters of the lesions. The results indicated the predominance of lesions in male young patients, with femoral location, most tumors being represented by the osteoblastic type, with high grade, size <8 cm and in advanced stages. Reactions were present in all cases, the high immunoeexpression being associated with osteoblastic/epithelioid types (Ki67, cyclin D1, p53), high grade (Ki67, cyclin D1) and advanced stage (Ki67, cyclin D1). The study revealed a positive linear relation of the investigated immunomarkers expression, which indicates their usefulness in identifying lesions with aggressive progression potential.

Keywords: osteosarcoma, Ki67, p53, cyclin D1.

Introduction

Osteosarcoma is the most common primary malignant tumor of bone tissue, which often has an aggressive evolution, with a 5-year survival rate varying between 10–70%, depending on the presence of metastases [1–5]. Data from the literature indicate that the development of osteosarcomas is a multifactorial and multistage process, the improved prognosis being associated with early diagnosis and rapid therapeutic intervention [5]. Although the prognosis of localized disease has been attenuated by improved surgical techniques and chemotherapy, the quality and lifespan of patients remains profoundly altered, largely due to a lack of knowledge of the biomolecular mechanisms involved in tumor progression [1, 6]. One of the most studied molecular mechanisms involved in the progression of osteosarcomas is represented by cell proliferation and factors that interfere with the cell cycle. Thus, over time, studies related to the immunoeexpression of Ki67, p53, p27, cyclin D1 were performed, which indicated some relations with the prognostic parameters of the lesions represented by the size, grade, and tumor stage [1, 5, 7]. However, studies related to the immunoeexpression of these proteins in osteosarcomas are relatively rare, and the results are sometimes controversial, especially due to the methodology or clinicopathological classifications of tumors.

Aim

In this study, we analyzed the immunoeexpression of Ki67, p53 and cyclin D1 for 18 cases of primitive osteosarcomas, in relation to the main clinicopathological prognostic parameters of the lesions.

Materials and Methods

The study included 18 cases of primitive osteosarcomas from patients investigated and operated in the Clinic of Orthopedics, Emergency County Hospital, Craiova, Romania, during 2010–2020. The tumors were diagnosed and evaluated in the Department of Pathology of the same Hospital, using the most recent criteria elaborated by American Joint Commission on Cancer (AJCC) and World Health Organization (WHO) [8, 9]. The biological material was represented by biopsy or radical tumor resection specimens, which were fixed in 10% neutral buffered formalin, decalcified, processed by classical paraffin embedding and Hematoxylin–Eosin (HE) staining.

The clinicopathological analysis followed the distribution of cases by age and gender groups, location, histological type (including histological differentiations), tumor grade, tumor extension (size/pT) and tumor stage established in clinical-imaging context, parameters that were analyzed in the report with the immunoeexpression of Ki67, p53 and cyclin D1 (Table 1).

The immunohistochemical (IHC) technique was done on serial 4 µm sections, in sequential steps consisting in alcohol rehydrating, endogenous enzyme blocking with 3% hydrogen peroxide, unspecific antigenic sites blocking with bovine serum albumin (BSA), respectively incubation with primary antibodies at 4°C overnight. The working system...
was represented by EnVision™ FLEX+ System (code K8002, Dako), and for visualizing the reactions we used 3,3’-Diaminobenzidine (DAB) tetrahydrochloride as chromogen. For the validation of IHC reactions, we used external positive and negative controls.

Table 1 – Antibodies used and immunostaining protocol

| Antibody | Clone | Dilution | Pretreatment | External positive control |
|----------|-------|----------|--------------|---------------------------|
| Ki67     | MIB-1/Dako (mouse monoclonal antihuman) | 1:100 | Microwaving in citrate buffer, pH 6 | Palatine tonsil |
| p53      | DO-7/Dako (mouse monoclonal antihuman) | 1:50 | Microwaving in Tris-EDTA buffer, pH 9 | Colon adenocarcinoma |
| Cyclin D1 | EP12/Dako (rabbit monoclonal antihuman) | 1:50 | Microwaving in Tris-EDTA buffer, pH 9 | Breast carcinoma |

EDTA: Ethylenediaminetetraacetic acid.

The semiquantitative assessment of the immunoreactions was performed using the positivity index (PI) expressed as a percentage of the number of positive cells reported to the total number of cells existing on microscopic field of 20×, for each case being counted five fields with maximum imaging, and pathological in tumor stage IIA (61.1%) (Table 2).

Table 2 – Distribution of cases in relation to the analyzed parameters, PI values of Ki67, cyclin D1 and p53 and the associated statistical significance

| Parameter / No. of cases | Ki67 PI / p-value | Cyclin D1 PI / p-value | p53 PI / p-value |
|--------------------------|-------------------|------------------------|------------------|
| Age / [years]            |                   |                        |                  |
| ≤25 / 12                 | 24.5±7.8          | 0.816                  | 2.5±1.6          | 0.504 |
| >25 / 6                  | 23±15             | 11±3.9                 | 8±4              |                  |
| Gender                   |                   |                        |                  |
| Male / 15                | 24.5±11.2         | 0.407                  | 10.2±4.5         | 0.230 |
| Female / 3               | 21.6±2.8          | 9.3±1.1                | 21               |                  |
| Location                 |                   |                        |                  |
| Femur / 10               | 21.5±5.7          | 0.405                  | 10.1±2.6         | 0.370 |
| Tibia / 6                | 28.8±16.1         | 9.5±6.2                | 8.5±4            |                  |
| Humerus / 2              | 22.5              | 1.5                    | 1.5              |                  |
| Histological type        |                   |                        |                  |
| Osteoblastic / 10        | 23.5±4.7          | <0.001*                | 3.2±1.4          | 0.010* |
| Fibroblastic / 4         | 21.2±2.5          | 10.7±2.9               | 1                |                  |
| Epithelioid / 2          | 47.5              | 11                     | 7.5              |                  |
| Low-grade / 2            | 9                 | 3                      | 1                |                  |
| Tumor grade              |                   |                        |                  |
| Grade 1 / 2              | 26.6±2.8          | 0.084                  | 4.3±1.1          | 0.329 |
| Grade 2 / 3              | 25.7±10.3         | 10.6±1.1               | 0.026*           |                  |
| Tumor size / extension (pt) |                  |                        |                  |
| T1 / 13                  | 23.6±12.1         | 0.729                  | 9.5±4.8          | 0.947 |
| T2 / 5                   | 25.3±5            | 11.4±2.1               | 3±2              |                  |
| IA / 2                   | 9                 | 3                      | 1                |                  |
| Tumor stage              |                   |                        |                  |
| IIA / 11                 | 26.3±11.2         | 0.082                  | 10.7±3.9         | 0.491 |
| IIB / 5                  | 25.3±5            | 11.4±2.1               | 3±2              |                  |

PI: Positivity index; pT: Primary tumor. *p-value: one-way analysis of variance (ANOVA) or Student’s t-test.

Osteoblastic osteosarcomas indicated the presence of osteoid, irregular bone trabeculae with intertrabecular spaces full of atypical tumor cells, with hyperchromatic nuclei and mitotic activity (Figure 1A). In comparison, in the case of fibroblastic osteosarcomas, the amount of osteoid was minimal, with atypical elongated or oval tumor cells, frequently with hemangiopericytoma-like architecture (Figure 1B), while epithelioid osteosarcomas consisted of plasmacytoid, sometimes polygonal cells with vesicular nuclei and visible nucleoli (Figure 1C). All the conventional carcinomas in this study had the tumor grade 2 or 3. In the case of central low-grade osteosarcoma, anastomosed bone trabeculae and elongated monomorphic cells with minimal atypia were present (G1) (Figure 1D).

In this study were included only primitive osteosarcomas, without history of systemic, oncological, or hormonal treatments and no history of cancer with other locations.

The statistical analysis used the comparison tests represented by one-way analysis of variance (ANOVA) or Student’s t-test and Pearson’s test within Statistical Package for the Social Sciences (SPSS) 10 software, the results being considered significant for values of p<0.05. For the images acquisition, we used the Nikon Eclipse E600 microscope equipped with Lucia 5 software.

The local Ethics Committee approved the study, which was done with the informed consent of the patients.

Results

The analysis of clinicopathological data for the 18 osteosarcomas included in the study indicated the predominance of tumors in patients aged ≤25 years (66.7%), males (83.3%), most being located at the femoral level (55.6%). The age of the patients ranged between 10–55 years, with a mean diagnostic age of 22.5±12.7 years. Conventional osteoblastic osteosarcomas (55.6%) predominated, followed by the fibroblastic (22.2%), epithelioid (11.1%) and low-grade central (11.1%) ones, most being of high-grade (G3) (88.9%), with size ≤8 cm (T1) (72.2%) and classified clinically, imaging, and pathological in tumor stage IIA (61.1%) (Table 2).
Figure 1 – Osteosarcoma, HE staining, ×200: (A) Osteoblastic type; (B) Fibroblastic type; (C) Epithelioid type; (D) Central low-grade type.

Figure 2 – Osteosarcoma, Ki67 immunostaining, ×200: (A) Osteoblastic type; (B) Fibroblastic type; (C) Epithelioid type; (D) Central low-grade type.
In relation to the tumor grade, the G2/G3 osteosarcomas indicated higher values of Ki67 PI compared to G1, respectively of 26.6±2.8 / 25.7±10.3 and 9, while lesions with size >8 cm (T2) had Ki67 PI values superior compared to ≤8 cm (T1) ones, respectively 25±3.5 and 23.6±12.1 (Table 2). The highest Ki67 PI values were observed in tumors classified in stages IIA and IIB, with values of 26.3±11.2 and 25±3.5, compared to stage IA, with a value of 9 (Table 2).

Cyclin D1 immunoexpression was superior in osteosarcomas present in male patients ≤25 years old, with femoral localization (Table 2). The highest values of cyclin D1 PI were present in conventional osteoblastic and epithelioid osteosarcomas, with values of 11±4 and 11, followed by fibroblastic type, with an average value of 10.7±2.9 and central low-grade type with an average value of 3 (Figure 3, A–D) (Table 2). High-grade G2/G3 tumors showed mean PI values of 10.6±1.1 and 11±3.8 compared with G1, with a value of 3, while T2 lesions were superior to T1 as an immunomarker expression, with mean values of 11.4±2.1 and 9.5±4.6, respectively (Table 2). Thus, the tumors in stage IIB had a cyclin D1 PI value of 11.4±2.1, tumors in stage IIA of 10.7±3.9, and those in stage IA of 3 (Table 2).

The p53 immunoexpression and implicitly the mean values of PI were lower compared to Ki67 and cyclin D1. p53 PI values were slightly higher in male patients >25 years old and tibial location (Table 2). The highest p53 values were identified in conventional epithelioid osteosarcomas, with a value of 7.5, followed by osteoblastic ones with PI of 3.2±1.4 and fibroblastic and central low-grade types with values of 1 (Figure 4, A and B) (Table 2). Grade G2/G3 tumors indicated higher values compared to G1 lesions, respectively 4.3±1.1, 2.9±2.5 and 1, while T2 and T1 lesions showed similar values, respectively 3±2 and 2.9±2.5 (Table 2). Regarding the tumor stage, tumors in stage IIA/IIB showed similar mean PI values, of 3.2±2.6 and 3±2, compared to stage IA with PI value of 1 (Table 2).

The statistical analysis did not identify significant differences of Ki67, cyclin D1 and p53 PI in relation to age (p>0.05, Student’s t-test), gender (p>0.05, Student’s t-test) and tumor location (p>0.05, ANOVA test) (Table 2). The mean PI values were statistically significantly superior or at the limit of significance in epithelioid and osteoblastic osteosarcomas, compared with fibroblastic and central low-grade ones, both for Ki67 (p=0.001, ANOVA test), cyclin D1 (p=0.070, ANOVA test) and p53 (p=0.010, ANOVA test) (Table 2). High-grade osteosarcomas showed statistically significantly higher or at the limit of significance PI, compared to low-grade osteosarcomas in the case of Ki67 (p=0.084, Student’s t-test) and cyclin D1 (p=0.026, Student’s t-test) (Table 2). We did not find statistical associations of the analyzed immunomarkers in relation to the size of the tumors (pT) (p>0.05, Student’s t-test). Analysis of PI values in relation to tumor stage indicated significantly higher or at the limit of significance differences in IIA/IIB tumors compared to IA tumors for Ki67 (p=0.082, ANOVA test) and cyclin D1 (p=0.024, ANOVA test) (Table 2) (Figure 5, A–C). In this study, we found a superior concordance of cyclin D1 immunoe expressed in tumors with an advanced stage.

Analysis of the PI values of the analyzed immunomarkers indicated significant positive linear correlations between Ki67/cyclin D1 (p=0.025, Pearson’s test) and Ki67/p53 (p<0.001, Pearson’s test) and nonsignificant in the case of cyclin D1/p53 (p=0.168, Pearson’s test) (Figure 5D).
Discussions

Osteosarcoma is the most common malignant bone tumor in adults, with an increased frequency in young ages, the origin being represented mainly by the metaphysis of long bones [2, 5, 10]. Some studies indicate a bimodal incidence of tumors, respectively in adolescents and over 60 years old, and a relatively equal distribution by gender groups [11, 12]. The results of our study indicated the predominance of osteosarcomas in patients ≤25 years old (12 cases), males (15 cases), tumors being present in order of frequency in the femur, tibia and humerus.

Some authors have indicated tumor size, histological grade, and the presence of metastases as the main prognostic factors for osteosarcomas, while others have shown the importance for survival of demographics, sensitivity to treatment and tumor location [1, 13]. At the same time, in the case of localized disease, the 5-year survival rate is 55–75%, with recurrences being present in 30–40% of cases [14]. In this study, the osteoblastic osteosarcomas predominated (10 cases), with fibroblastic and epithelioid tumors being present, as well as central low-grade osteosarcomas, most of which were of high-grade (G2/G3, 16 cases), with size ≤8 cm (13 cases) and in advanced stages.
Age at diagnosis and histological subtype are controversial prognostic factors [15]. Thus, there are rare studies related to the involvement of the tumor type in establishing the prognosis of patients, which indicated the absence of differences between the conventional telangiectatic and fibroblastic osteosarcomas, although the chondroblastic ones are easier to manage [15]. On the contrary, other studies indicate a higher aggressiveness and recurrence of non-osteoblastic osteosarcomas compared to osteoblastic ones [16].

In the context of lesion prognosis, the analysis of the biomolecular mechanisms involved in the progression of osteosarcomas is current in research. There were numerous prognostic factors for osteosarcomas represented by leucine-rich repeat containing 15 (LRRC15) membrane protein, transferin receptor 1, vascular endothelial growth factor (VEGF), but which did not correlate with most tumor prognostic factors – hence the need to identify such a marker [11].

One of the most studied biomolecular mechanisms in osteosarcoma is the cell proliferation [5]. In this context, one of the immunomarkers frequently investigated in osteosarcomas is Ki67, which is a nonhistonic protein that includes two isoforms, which is present in cells in the G1, S and G2 phases of the cell cycle and mitosis but absent in cells in rest or in the G0 phase, which proves involvement in cell proliferation [17]. Ki67 immunoreactions are correlated with the prognosis of many malignancies with different locations, although the role of the protein appears to be much more complex, with variations in cell distribution during interphase and mitosis [18]. In a study by Mardanpour et al., which included 56 osteogenic osteosarcomas, Ki67 immunoreactions were present in 96.5% of cases and correlated with clinicopathological prognostic factors represented by size, tumor grade and the presence of lymph node metastases, as well as low survival rate [1]. While there are studies that correlate Ki67 with tumor grade [19], there are also studies that contradict these results [20]. Metastasis issues are also controversial.

In our study, Ki67 reactions were present in all cases, the increased values of the immunoreactions being associated with the epithelioid subtype, as well as the high-grade and advanced stage of tumors.

Studies are controversial about the significance of Ki67 in bone tumors, while some studies indicate an association with aggressivity or survival, others indicate the absence of such relations [21, 22]. In the case of osteosarcoma, the overall results are inconsistent, so the role and significance of Ki67 is still unclear. Also, the positivity of Ki67 seems irrelevant for the classification of osteosarcomas [11].

Cyclin D1 plays an important role in cell proliferation, representing the link between extracellular environment signaling and cell cycle progression [23, 24]. Cyclin D1 binds to cyclin-dependent kinase 4 or 6 for the formation of an active kinase complex that phosphorylates the retinoblastoma inhibitory protein, allowing E2F transcriptional factor to promote cell cycle progression [23, 25]. Although it is an unstable protein, with a half-life of 24 minutes, being degraded in a ubiquitin-dependent manner, it also has an independent role as a modulator of some transcriptional factors [26, 27]. There are studies that have shown that overexpression of cyclin D1 reduces the G1 phase of the cell cycle, progression to the S phase, and deoxyribonucleic acid (DNA) duplication [5, 28]. The expression increases under the stimulation of the cells entering the cell cycle, being proposed the transfer to and from the nucleus during the cell cycle of the active cells [23, 29].

Cyclin D1 is a regulatory protooncogene of the cell cycle, its overexpression being present in many primary human cancers (squamous cell carcinomas of the neck, hepatocellular carcinoma, esophageal, pulmonary, urothelial, or mammary gland carcinomas – where it induces resistance to hormone therapy), useful in diagnosis and prognosis assessment [5, 26, 30]. Overexpression of cyclin D1 in cancers is not due to gene amplification, but to post-translational disturbance, experimental studies on tumor cell cultures indicating increased expression of cyclin D1 in association with the initiation of neoplastic processes, especially by stimulating cell proliferation [26, 31]. In the study by Wu et al., the positivity rate of cyclin D1 in osteosarcomas was 73%, compared to 3.3% in benign lesions, and no reactions in normal tissues [5]. At the same time, cyclin D1 appears to activate the mechanisms of transformation and proliferation of osteoblasts in osteosarcomas [32]. The relation of cyclin D1 immunoreexpression with histological prognostic factors of osteosarcomas is practically nonexistent in the literature. In our study, the high immunoreactions of cyclin D1 were associated with conventional types, as well as advanced tumor grade and stage.

p53 is an important tumor suppressor and a central mediator of the cellular response, with mutations with the effect of gene inactivation being present in 50% of human cancers [1, 33]. As with other tumors, p53 mutations in osteosarcomas induce significant genomic instability [1, 34].

In the study by Mardanpour et al., the positivity rate of cyclin D1 in osteosarcomas was present in 89% of categories, correlating with the prognostic factors of osteosarcomas represented by tumor grade, size, presence of metastases, but without correlation with survival at three or five years [1]. The association of p53 positivity with high tumor grade osteosarcomas is also indicated in other studies [35]. In our study, the highest values of p53 immunoreexpression were associated with osteoblastic and epithelioid types, and without statistical relation with the other clinicopathological parameters analyzed. At the same time, there are studies performed on cell lines that have shown an increase in p53 immunoreexpression in the case of low-grade osteosarcomas, Without metastases and with a better prognosis [36].

**Study limitations**

The controversial results in the literature of the expression of the immunomarkers investigated in this study may be due to the low number of cases, methodology and clinicopathological classifications of the lesions. The study can be considered limited by the relatively small number of cases, on the one hand due to the low incidence compared to other malignancies and on the other hand due to the strict application of inclusion criteria to have a homogeneous investigation group, to objectively quantify the expression of immunomarkers. In this context, future studies are needed to validate the results obtained on large groups.
The results of the study indicated significant differences in the expression of the analyzed immunomarkers in relation to the tumor type, the highest PI values being recorded for conventional osteosarcomas respectively for epitheloid and osteoblastic types. Ki67 and cyclin D1 immunoexpression was superior in the case of high-grade tumors, with extension and advanced stage, the immunomarkers proving their usefulness for identifying potentially aggressive lesions, to stratify patients for optimal targeted therapy.

Conflict of interests

The authors declare that they have no conflict of interests.

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