Immuno Histochemical Study of Osteosarcoma Using Ki-67 as a Labeling Index in Naturally Fluoridated Community

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Abstract

Osteosarcoma is the most common primary malignant bone tumor which characterized microscopically by formation of osteoid or bone by the neoplastic cells (osteoblastic type) or production of fibrous tissue (fibroblastic type) and/or a cartilage (chondroblastic type). Ki-67 protein is present during all active phases of the cell cycle (G1, S, G2 and M) but is absent from resting cells (G0), makes it an excellent marker for determining the so called growth fraction of a given cell population. However, fluoride concentration in drinking water shows great variation between the coastal-western region of Libya (mean fluoride concentration 1.38ppm) and Cairo-Egypt (0.4ppm). Therefore, the aim of this study is to evaluate and compare histopathologically the proliferative activity of Osteosarcomas in naturally fluoridated community (Libyan cases) and non-fluoridated community (Egyptian cases) as a retrospective case-control study using the labeling index Ki-67. 20 cases of Osteosarcoma from each country in a formalin-fixed embedded specimens and serial sections from the paraffin blocks for immuno histochemical examination were included in this study in which Peroxidase-antiperoxidase technique was performed. Assessment of Ki-67 proliferative activity was carried out using software image analysis and the area fraction was given by the percentage of immuno positive area to the total area of the microscope field. The result of this study showed that the mean value of area of fraction in Libyan cases was 5.33 and in Egyptian cases were 3.12. When comparing the area fraction of Ki67 immunopositivity of tumor cells in Lybian cases versus Egyptian cases, statistical analysis revealed a statistically significant difference (P value=0.023). It is concluded from this study that a high expression of Ki-67 of Osteosarcomas in fluoridated area compared to those of non-fluoridated area could explain the aggressive behavior of this tumor, in which fluoride ions may play a role as an underlying etiological factor.

Keywords: Osteosarcoma; Peroxidase-antiperoxidase Technique; Ki-67; Area of Fraction; Fluoride.

Introduction

Osteosarcoma is the most common primary malignant bone tumor [1], by definition, an Osteosarcoma is a neoplasm in which osteoid is synthesized by malignant cells. Conventional Osteosarcoma accounts for 90 % of all Osteosarcomas. It begins in the medullary canal and sometimes penetrates the cortex and invades the adjacent soft tissues [2]. Osteosarcomas are sub classified by WHO into many variants that differ in location, clinical presentation and histopathological features such as conventional Osteosarcoma, osteoblastic, chondroblastic and fibroblastic [3]. Osteosarcoma, usually affects children and young adults with estimated incidence of 4-5 per million populations. It most frequently occurs in the second decade of life with some 60% of patients under the age of 25 years, although 30% of osteosarcomas occur in patients over 40 years of age [4,5]. In younger patients, Osteosarcoma most commonly occurs in the long bones, in particular, the distal femur, proximal tibia, proximal humerus and around the knee [6,7]. By contrast, older patients tends to develop osteosarcoma more frequently in non-long bones such as jaws [8], pelvis, spine and skull, while osteosarcoma arising in bones distal to the wrists and ankles is extremely rare [9,10]. Pain with or without a palpable mass, tenderness, or swelling rarely occurring for more than a few months, are the usual presenting symptoms. Pain is usually deep, boring and severe; other findings may include limitation of normal function, edema, localized warmth, tellangectasias and pathological fracture [10,11]. The overall radiographic features of osteosarcoma are variable and depend on the amount of normal bone destroyed by the tumor and the amount of neoplastic bone formed within the lesion. The lesion may be purely osteoblastic, osteolytic or mixed lytic/blastic lesion. When the cortical plates are perforated, the periosteum is raised and the tumour may extend into the surrounding soft tissue to produce the classical sun-ray appearance [12,13]. Gross examination of osteosarcoma is often showing a large tumor, usually over 5 cm, fleshy or hard in consistency which may contain osteoid, fibrous tissue...
or cartilage. It frequently perforated the cortex and associated with a soft tissue mass. Some osteoblastic variety may appear grey-tan or pumice-like in color; while others become, sclerotic and more yellow-white. Chondroblastic variety tends to be white to tan in color; and variably calcified with a fish-flesh or rope-like cut surface [14]. Microscopically, Osteosarcomas show considerable variation in pattern, but by definition, the tumor is characterized by formation of osteoid or bone by the neoplastic cells (osteoblastic type) or production of abundant fibrous tissue (fibroblastic type) and/or a cartilage (chondroblastic type). In osteoblastic osteosarcoma, bone and/or osteoid are the predominant matrix, the pattern and the distribution of osteoid is often variable, in some areas, osteoid is deposited in fine lace-like seams, in other areas broad sheets of osteoid may entrap single neoplastic osteoblasts [15,16]. In addition to osteoblastic areas, other patterns such as chondroblastic differentiation are often present, in which chondroid matrix is predominant, which contain lobules or islands of cartilage with markedly atypical chondrocytes. Myxoid and other forms of cartilage are uncommon except in the jaws and pelvis. A third pattern of differentiation that may be focally present resembles fibro sarcoma or malignant fibrous histiocytoma is the fibroblastic osteosarcoma, which is a high grade spindle cell malignancy, these atypical spindle cells are often arranged in a storiform pattern admixed with tumour giant cells with only minimal amounts of osteoid with or without cartilage [17].

The precise etiology of OS remains unknown due to lack of understanding of the cell or origin, the absence of identifiable precursor lesions, and its marked genetic complexity at the time of presentation. Interestingly, several human genetic disorders, familial cancer syndromes such as Li-Fraumeni syndrome, history of trauma, Paget’s disease of bone, fibrous dysplasia, osteoblastoma, osteochondroma and radiation exposure and recently the high-fluoride level in drinking water are all linked to an increase risk of development of Osteosarcoma [18-22]. Cytogenetic studies of osteosarcomas showed that the most, if not all, osteosarcomas have clonal chromosomal aberration either numerical or structural. Multiple clones are common and diploid ploidy pattern by DNA cytofluorometry has been reported to be a poor prognostic sign [23,24]. Although, no specific translocation or other diagnostic structural aberration has been assigned to conventional Osteosarcoma. However, there is recurrent involvement of certain chromosomal regions such as 1p11-13, 1q11-12, 1q21-22, 11p14-15, 14p11-13, 15p11-13, 17p and 19q13 are most frequently affected by chromosomal structural aberration and the most common imbalances are +1, -6q, -9, -10, -13 and -17 as well as cytogenetic manifestations of gene amplification are frequently seen [25,26].

Ki-67 Protein and Cellular Division

Antigen Ki-67 also known as Ki-67 or MKI67 is a protein that in humans is encoded by the MKI67 gene located on chromosome 10. Ki-67 protein was originally defined by the prototype monoclonal antibody Ki-67 which was generated by immunizing mice with nuclei of Hodgkin lymphoma cell line L428. The name is derived from the city of origin (Kiel, Germany) and the number of the original clone in the 96-well plate [27]. Ki-67 protein is a cellular marker for proliferation. It is strictly associated with cell proliferation. During the interphase, the Ki-67 antigen can be exclusively detected within the cell nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosome. Ki-67 protein is present in during all active phases of the cell cycle (G1, S, G2, and mitosis), but is absent from resting cells (G0), makes it an excellent marker for determining the so called growth fraction of a given cell population [28,29].

Materials and Methods

Fourthly cases formalin-fixed, paraffin embedded specimens of osteosarcoma were included in this study. Twenty cases were collected from different Libyan Hospitals (Pathology Departments of Sabrata Tumor Institute and Tripoli Medical Center), between the years 1998 and 2008. On the other hand, twenty cases of osteosarcomas were collected from Cairo Hospitals (Pathology Departments of Ain Shams Specialized Hospital and National Cancer Institute between the years 2006 and 2009. The Osteosarcoma cases from both countries were further subdivided according to the histological features into osteoblastic, chondroblastic and fibroblastic variants. In order to confirm the diagnosis of all the cases and determine the histological variant of the cases included in this study. Two slides from each case were stained again with Haematoxyline and eosin and re-examined by two pathologists from Pathology Department, Faculty of Medicine, and Ain Shams University. Data collected and tabulated according to the diagnosis and sub variation of the tumor (Table 1). For all the cases of osteosarcomas, Peroxidase-antiperoxidase technique was performed for the immuno histochemical staining using the ready-to-use universal Kit, Ultra Vision Detection System Anti-Polyvalent, Horse Raddish Protein (HRP), purchased from Lab Vision Corp., USA in which primary antibodies: For detection of Ki-67 protein, a monoclonal mouse pre diluted anti-Ki-67 antibodies (clone MIB1) was used to react with the protein product of the Ki-67 gene.
Table 1: Histopathological data of Libya and Egyptian cases

| Case No. | Diagnosis | Histological Variant | Case No. | Diagnosis | Histological Variant |
|----------|-----------|----------------------|----------|-----------|----------------------|
|          |           |                      |          |           |                      |
| Case No. |           |                      | Case No. |           |                      |
| 1        | Osteosarcoma | Osteoblastic         | 1        | Osteosarcoma | Osteoblastic         |
| 2        | Osteosarcoma | Osteoblastic         | 2        | Osteosarcoma | Osteoblastic         |
| 3        | Osteosarcoma | Osteoblastic         | 3        | Osteosarcoma | Osteoblastic         |
| 4        | Osteosarcoma | Osteoblastic         | 4        | Osteosarcoma | Osteoblastic         |
| 5        | Osteosarcoma | Osteoblastic         | 5        | Osteosarcoma | Osteoblastic         |
| 6        | Osteosarcoma | Osteoblastic         | 6        | Osteosarcoma | Osteoblastic         |
| 7        | Osteosarcoma | Osteoblastic         | 7        | Osteosarcoma | Osteoblastic         |
| 8        | Osteosarcoma | Osteoblastic         | 8        | Osteosarcoma | Osteoblastic         |
| 9        | Osteosarcoma | Osteoblastic         | 9        | Osteosarcoma | Osteoblastic         |
| 10       | Osteosarcoma | Osteoblastic         | 10       | Osteosarcoma | Osteoblastic         |
| 11       | Osteosarcoma | Osteoblastic         | 11       | Osteosarcoma | Osteoblastic         |
| 12       | Osteosarcoma | Osteoblastic         | 12       | Osteosarcoma | Chondroblastic       |
| 13       | Osteosarcoma | Osteoblastic         | 13       | Osteosarcoma | Chondroblastic       |
| 14       | Osteosarcoma | Chondroblastic       | 14       | Osteosarcoma | Chondroblastic       |
| 15       | Osteosarcoma | Chondroblastic       | 15       | Osteosarcoma | Chondroblastic       |
| 16       | Osteosarcoma | Chondroblastic       | 16       | Osteosarcoma | Fibroblastic         |
| 17       | Osteosarcoma | Chondroblastic       | 17       | Osteosarcoma | Fibroblastic         |
| 18       | Osteosarcoma | Chondroblastic       | 18       | Osteosarcoma | Fibroblastic         |
| 19       | Osteosarcoma | Chondroblastic       | 19       | Osteosarcoma | Chondroblastic       |
| 20       | Osteosarcoma | Chondroblastic       | 20       | Osteosarcoma | Chondroblastic       |

**Immunostaining procedures**

For all specimens, 2 sections five-micrometer each, were mounted on positively charged slides to be immunostained with anti-Ki67 antibody. Sections were put in xylol overnight, then in the hot oven at 65°C for one hour and a fresh xylol wash was done for absolute deparaffinization. For rehydration, the sections were embedded in descending concentrations of alcohol: 100%, 85%, 75%, then 50%, for 5 min each, then washed in running water for 10 min. Afterwards, the sections were washed in two PBS preparations for 3 minutes each. The excess fluid was blotted around the tissues with filter paper. For heat induced epitope antigen retrieved (HIER), the sections were immersed in citrate buffer solution of pH 4.8. Sections immersed in the retrieval were put in the microwave oven for approximately 5-7 min, till the point of start boiling, after which they were left to bench cool for 20 min and then washed in a bath of distilled water for 10 min. Sections were washed in two PBS preparations for 3 minutes each. The excess fluid was blotted around the tissues with filter paper. For heat induced epitope antigen retrieved (HIER), the sections were immersed in citrate buffer solution of pH 4.8. Sections immersed in the retrieval were put in the microwave oven for approximately 5-7 min, till the point of start boiling, after which they were left to bench cool for 20 min and then washed in a bath of distilled water for 10 min. Sections were washed in two PBS preparations for 3 minutes each. The excess fluid was blotted around the tissues with filter paper. Two to three drops of primary ready-to-use antibody were added (Monoclonal mouse anti-Ki67 antibody) and incubated overnight in a humidity chamber at room temperature. Sections were washed in two PBS preparations for 3 minutes each. Enzyme conjugate was applied to the sections to completely cover them, for 10 minutes at room temperature. Sections were washed in two PBS preparations for 3 minutes each. The excess fluid was blotted around the tissues with filter paper then sections mounted with the Canada balsam mount and finally a cover was put on the slides.

**Assessment of Ki-67 proliferative activity**

Areas from each section composed predominantly of tumor tissue with the highest proliferative activity (brownish immunopositive cells) were selected and areas of reactive bone, fibrosis, hemorrhage and inflammation were preferentially excluded. For each section, at least three microscopic fields showing areas of highest Ki-67 expression were photographed at a magnification (x100), using a digital video camera (Olympus, C5060, Japan). The camera was mounted on a light microscope (Olympus, BX 60, Japan). Images were then transferred to the computer system for further adjustment and analysis. Assessment of Ki-67 proliferative activity was carried out using software image analysis (Image J, 1.41 a, NIH, USA). Automatic correction of images for brightness and contrast was performed first, while Phase analysis was calculated automatically giving...
the area fraction of the positive nuclei (Figure 1). The area fraction was given by the percentage of immunopositive area to the total area of the microscope field. Finally, the area fraction for each microscopic field was calculated to be used in statistical analysis. Data was collected and tabulated using Microsoft Excel 2003 and then statistically analyzed using SPSS version 15. Independent samples T-test used as a pair-wise test for assessment of mean area fraction of Ki-67 immunopositivity in Libyan versus Egyptian cases. All statistical analysis performed at the significance level if P value < 0.05.

### Immunohistochemical Results

Immunostaining of tumor tissue by anti-Ki67 monoclonal antibody revealed a positive nuclear immuno staining of tumor cells. The reaction was brownish and granular. In Libyan cases, both osteosarcoma and chondrosarcoma tumor cells showed positive nuclear or nuclear/cytoplasmic immunopositivity (Figures 2-3). Determination of immunopositive reaction by estimation of area fraction of immunopositivity (% of immunopositivity in a microscopic field at a definite magnification) revealed a mean value 5.3355 in all Libyan cases (Table 2). On the other hand, in Egyptian cases, osteosarcoma, chondrosarcoma and plasmacytoma tumor cells showed positive nuclear or nuclear/cytoplasmic immunopositivity (Figures 4-5). Determination of immunopositive reaction by estimation of area fraction of immunopositivity (% of immunopositivity in a microscopic field at a definite magnification) revealed a mean value 3.122 in all Egyptian cases (Table 2). When comparing the area fraction of Ki67 immunopositivity of tumor cells in Libyan cases and Egyptian cases, statistical analysis revealed a statistically significant difference (P value = 0.023) (Table 3).

| Group     | N  | Mean | Std. Deviation | Std. Error Mean |
|-----------|----|------|----------------|-----------------|
| Area Fraction |    |      |                |                 |
| Egyptian  | 20 | 3.122| 3.02614        | 0.67667         |
| Lybian    | 20 | 5.3355| 2.89393       | 0.6471          |

**Figure 1**: Steps of image analysis for calculation of area fraction of Ki67 immunopositivity

**Figure 2**: Photomicrograph of Osteosarcoma of Libyan patient showing positive nuclear Ki67-immunopositivity in tumor cells. (Ki67, x200)

**Figure 3**: Photomicrograph of Osteosarcoma of Libyan patient showing positive nuclear Ki67-immunopositivity in tumor cells. (Ki67, x100)
Discussion

Since fluoride acts as an anabolic agent for bone cells and that its osteogenic properties are mediated primarily by an increase in osteoblastic proliferation, as well as most of the fluoride is deposited in actively mineralizing areas of bone [30-32] and cartilage [33,34]. Although, the mitogenic action of fluoride and genotoxicity action (ability of fluoride to induce chromosomal aberrations) was subsequently confirmed by a number of laboratories on bone cells of various species, including humans [35-38]. Therefore, it is biologically plausible that widespread exposure to fluoride during critical periods of growth may play a role in the etiology of OS, but still there are conflicting data regarding the association between fluoride exposure and the incidence of osteosarcoma. Several human and animal studies have been conducted in which many human epidemiological studies and animal cancer bioassay show a strong association between fluoride exposure in drinking water and the incidence of Osteosarcoma [5,19,21,39-43]. Adding to that, a very recent study found that, fluoride level in the neoplastic tissue of Osteosarcomas and the osteoblastic variants of this malignant tumor are significantly higher among the cases collected from regions where the fluoride level in drinking water is higher than the normal limit that recommended by WHO [22]. But, however, in contrast to these studies, many other studies have failed to find this link [44-46]. Fluoride concentration in public drinking water shows great variation between the costal-western region of Libya (mean fluoride level 1.38 ppm) and Cairo-Egypt (fluoride level 0.4 ppm) [47]. Therefore, this study was performed in these two different communities (Libya and Egypt), as a case-control retrospective study of Osteosarcoma using monoclonal antibodies against Ki-67 protein as a parameter to detect proliferating cells of malignant osteoblasts and to analysis the variation. Ki-67 protein is present during the active phases of the cell cycle (G1, S, G2 & mitosis) but is absent from resting cells G0, makes it an excellent marker for determining the so called growth fraction of a given cell population. It was first described in 1983, by Gerdes et al. [27,48,49] to be used on fresh tissue. With the introduction of the MIB-1 antibody, a true Ki-67 equivalent, and a new antigen retrieval technique with the use of microwaves, immunostaining of formalin-fixed paraffin-

Table 3: Independent samples T-test for equality of means of area fraction of Ki67 immunopositivity in Lybian and Egyptian cases.

| Levene’s Test for Equality of Variances | t-test for Equality of Means | 95% Confidence Interval of the Difference |
|----------------------------------------|----------------------------|----------------------------------------|
| F                                      | Sig.                      | T                                      | Df | Sig. (2-tailed) | Mean Difference | Std. Error Difference | Lower | Upper |
| Equal variance assumed                 | 0.062                     | -2.364                                 | 38 | 0.023          | -2.2135         | 0.93628              | -4.109 | -0.3181 |
| Equal variance not assumed             | -2.364                    | 37.924                                | 0.023          | -2.2135         | 0.93628              | -4.10902            | -0.31798 |
embedded material was possible with a high reproducibility, equaling results obtained. Unfortunately, little data exist on the use of Ki-67 in osteosarcoma. Although, the reliability of Ki-67 immunostaining has been evaluated in different tumour types. Ki-67 was lower in benign and low grade lesions as compared with high-grade malignant tumours. Alterations in Ki-67 were also used to identify the potential of proliferative marker as predictor for metastases in osteosarcoma patients [50]. The best studies examples in this context are carcinomas of the prostate, brain and the breast. For these types of tumors, the prognostic value for survival and tumour recurrence have repeatedly been proven in uni-and multivariate analysis. The preparation of new monoclonal antibodies that react with the Ki-67 equivalent protein from rodents now extended the use of Ki-67 protein as proliferative marker to laboratory animals that are routinely used in basic research as well as computerized image analysis, which is the main method of assessment used in this study, increases reproducibility, minimize inter-operator variability and decrease operator time in quantifications of immuno stained tissue sections [48,50]. In this study, detection of Ki-67 is carried out on biopsies samples of tumour cells using this special technique. The histological sections incubated with antibodies that can react with the Ki-67 molecule and then treated with special reagents that cause a color to appear where antibody has bound. This molecule can be detected in the nucleus of only actively growing cells, therefore the number of the tumour cells that are actively dividing, obtained through this examination is termed the S-phase, growth, or proliferative fraction and cells were considered immunopositive when even minimal brown staining was detected regardless of the intensity of brown color. In Libyan cases, this study showed that, all bone tumors revealed a high proliferative activity of Ki67 expression ranges between 4-6.8 with mean 5.33 and Std. Deviation 0.6. On the other hand, the Egyptian cases showed low Ki67 expression, only four cases demonstrated high reaction while other cases showed moderate Ki-67 expression. The image analysis ranges between 1.8-4.6 with a mean of 3.02 and std Deviation 0.67. In the present study, a highly significant Ki-67 positivity among the Libyan cases indicating a high-grade osteosarcoma (means a poor prognosis but there is no relevant clinical data regarding prognosis). There was an increase in the number of immuno positive cells with loss of differentiation in the tumor cells. Thus Ki-67 can be considered as a reliable and easy mean of accurately assessing the growth fraction of human osteosarcoma. It does appear to provide valuable diagnostic information. Moreover, an underlying etiological factor such as the mitogenic action of fluoride may play a role in increasing the proliferative activity of malignant osteoblasts towards the production of malignant osteoblastic osteoid tissue rather than the chondroblastic or fibroblastic tissue. However, there is no any relevant data regarding study of Osteosarcoma using Ki 67 in naturally fluoridated districts compared to non-fluoridated areas, therefore, this research may be regarded as the first leading research in this aspect. Katia et al. [54], showed that in bone tumors, the level of Ki-67 expression correlates with the level of malignancy and is diagnostically and prognostically useful while Tawfik et al. and Zhi et al. [55,56] demonstrate that metastatic OSs appears to have a significantly higher level of proliferative activity as compared to primary tumors and Ki-67 protein may be involved in the development, progression and metastasis of Osteosarcoma and is expected to serve as a new diagnostic and therapeutic target for osteosarcoma.

**Conclusion**

Based on the results of the present study, it may be concluded that, a high expression of Ki-67 of Osteosarcomas in fluoridated area compared to those in non-fluoridated area explains the aggressive behavior of osteosarcoma and fluoride ions has a mitogenic effect on malignant osteoblasts. Ki-67 could be used as a diagnostic marker in patients with osteosarcoma and may provide a better histological grade which could affect the prognosis and survival rate.

**Recommendations**

It is essential to consider remedial measures to control fluoride level. If fluoride levels of potable water were consistently beyond permissible levels. One possibility is to search for a safe water source locally or transported from distant safe sources through a piped water supply system. Defluoridation of water should be considered only where other options are not feasible or as an interim measures, if the other options take a long time for planning and implementation. The link between fluoride level in drinking water and osteosarcoma needs endless effort to achieve more progression in this field. It is essential to consider remedial measures to control fluoride level. If fluoride levels of potable water were consistently beyond permissible levels. One possibility is to search for a safe water source locally or transported from distant safe sources through a piped water supply system. Defluoridation of water should be considered only where other options are not feasible or as an interim measures, if the other options take a long time for planning and implementation. The link between fluoride level in drinking water and osteosarcoma needs endless effort to achieve more progression in this field.

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