



Bone Marrow Micrometastases Studied by an Immunomagnetic Isolation Procedure in Extremity Localized Non-metastatic Osteosarcoma Patients

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Abstract Hematogenous spread of tumor cells is an early event in osteosarcoma and present in the majority of patients at primary diagnosis. Eradication of such micrometastases by adjuvant combination chemotherapy is crucial for survival. However, a survival plateau of 60-70% was reached over two decades ago, above which it seems difficult to further advance with the currently available therapies.

In this study we have, by an immunomagnetic isolation procedure, examined the presence and prognostic impact of disseminated tumor cells in bone marrow aspirates taken at primary diagnosis in a cohort of 41 non-metastatic patients with extremity localized, high-grade osteosarcoma.

Introduction

One characteristic feature of osteosarcoma (OS) is the early hematogenous spread of tumor cells in a majority of patients. The successful eradication of micrometastases (MM) by adjuvant combination chemotherapy is crucial for survival.¹ A survival plateau of 60-70% was reached over two decades ago,² above which it still seems difficult to advance with the current diagnostic and therapeutic armamentarium.

OS displays considerable heterogeneity in metastatic capacity and chemosensitivity.²⁻⁶ Historical evidence has revealed that as many as 20% of OS patients without overt lung metastasis detected at primary diagnosis were in fact cured by surgery alone.^{2,7} In the group of patients who would otherwise relapse, approximately 50% have chemosensitive tumors and are cured by the adjuvant therapy.² It would be important to identify two subgroup of patients; i.e. (a) those not having MM disease – and spare them from the toxic post-operative adjuvant chemotherapy currently given to all, and (b) the cohort of 30-40% having chemore-

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sistant MM who currently succumb to their disease. In this group, the most aggressive combination of chemotherapy is justified, and novel therapies should be explored, ideally instituted already in the primary/neo-adjuvant setting.

Risk-adapted therapy and individualized treatment has significantly improved the outcome in other cancers.^{8,9} Unfortunately, it has thus far not been possible at the time of primary diagnosis to identify OS patients that belong to the different risk groups, and improved methods are needed to further advance the survival and/or reduce long-term toxicity from chemotherapy.

In several other cancers, the presence of MM; i.e. disseminated tumor cells (DTC) detected in bone-marrow (BM) or peripheral blood, is convincingly shown to have a negative prognostic impact.¹⁰⁻¹⁵ We have previously reported our first experience on DTC in 60 patients with primary bone sarcomas, 49 of whom had OS.¹⁶ In the present paper, we have updated the follow up time and disease related events among the 22 patients that presented with extremity localized, non-metastatic, high grade OS at clinical presentation in this first series. In addition, BM aspirates from another 19 OS-patients treated at our institution have been collected. Hence, in the current study we have examined the presence and prognostic impact of DTC in BM at primary diagnosis in a cohort of 41 patients with extremity localized, high-grade OS without evidence of metastases at primary diagnosis.

Background

It has long been a goal to identify MM in OS patients. In two theses from The Mayo Clinic, a tritiated thymidine labeling method was explored,^{17,18} and researchers at our institution used a technique employing Millipore filters in the vein draining the primary tumor.^{19,20}

More recently, improved methods have been developed to detect DTCs in several types of cancer.^{21,22} Our Institution has pioneered the development of an immunomagnetic procedure (Fig. 1) permitting rapid isolation of tumor cells present in samples of peripheral blood and BM aspirates from cancer patients.^{14,16,23,24}

To our knowledge, the only recent publication on DTC in OS is our first series of 60 patients with suspected bone sarcoma¹⁶ studied by the immunomagnetic detection assay mentioned above. Forty-nine of the patients had OS, and of these 63% had tumor cells in BM. Only four (8%) were positive in peripheral blood also. None of the 38 control BM samples were positive, including 11 from patients with suspected bone sarcoma at the time of sampling who later were found not to have OS.¹⁶ Among the 22 patients with extremity localized, non-metastatic, high-grade OS, none of the 10 DTC-negative patients did relapse, whereas four of the 12 DTC-positive did. Information was available on the histological response to pre-operative chemotherapy in 15 of these 22 patients. None of the three patients in the BM-negative group who had a poor response to chemotherapy did relapse, whereas two of the four poor responders in the BM-positive cohort, died of disease.¹⁶ We further characterized the immunomagnetically isolated cells by the use of fluores-

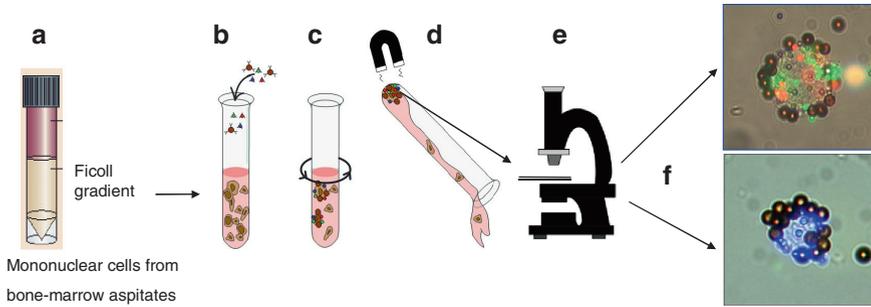


Fig. 1 Mononuclear cells are obtained by density gradient centrifugation of heparinized bone-marrow aspirates (A). Paramagnetic iron-containing monodisperse beads – Dynabeads[®] pre-incubated with monoclonal antibodies binding to cell surface markers are added (B). Incubation for 30 min on ice during constant rotation (C). A magnetic field is applied, and the supernatant is decanted (D). Rosettes are scored in a microscope (E). The technique also allows for detecting several cell surface epitopes simultaneously – by adding fluorescent latex-microbeads coated with other monoclonal antibodies that used for the rosetting process (F), see also ref.¹⁶

cent latex microparticles with surface-bound antibodies targeting different membrane markers (see Fig. 1F). In cases with numerous OS cells in BM, attempts to grow the isolated cells *in vitro* were successful in 2/8 attempts, and in 2/5 cases *s.c.* injected rosettes produced tumors with OS characteristics in nude mice,¹⁶ proving the malignant properties of the selected DTCs in these cases.

Materials and Methods

We have now obtained mononuclear cells (MNC) from iliacal crest aspirated bone-marrow, as previously described,¹⁶ in a total of 41 patients with localized extremity OS. See www.ssg-org.net for the consecutive clinical protocols SSG-II, SSG-VIII, ISG/SSG-I and SSG XIV describing the various chemotherapy combinations given.

Twenty-two were males and 19 were females. Anatomical sites of the primary tumor were: Femur – 19, tibia – 10, humerus – 10 and fibula – 2. All the patients were studied at primary diagnosis and were free of overt metastases as assessed by CT of the chest and ^{99m}Tc MDP bone scintigraphy. Patients with a minimum follow up of 2 years were included in this study.

Two monoclonal antibodies (Mabs) were used for the immunomagnetic study. TP-3 detects an epitope on an OS-associated cell surface antigen with homology to the bone isoenzyme of alkaline phosphatase.^{25–28} The high affinity Mab 9.2.27 (obtained from Dr. R. Reisfeld, Scripps Research Institute, La Jolla, CA) was originally developed against melanoma.²⁹ This Mab recognizes a cell surface epitope on the high molecular weight melanoma-associated antigen, and is also shown to bind some subgroups of sarcoma, including OS.³⁰ Both Mabs have previously been shown to be non-reactive with MNC in peripheral blood and bone marrow from normal donors.^{16,26,30}

The rapid and simple procedure for immunomagnetic detection of cancer cells in BM-samples has a sensitivity of approximately 2 target cells in 2×10^7 MNC, depending on the affinity of the monoclonal antibody used and the number of antigen epitopes expressed in a particular target cell population.²⁴ Briefly, iron containing, super-paramagnetic monodisperse particles with a diameter of 4.5 μm , coated with polyclonal sheep anti-mouse IgG (Dynabeads SAM-450, Invitrogen AS, Oslo, Norway), are pre-incubated with one of the tumor-associated Mabs and washed before the isolation procedure is performed (Fig. 1). Typically, 60 μg of purified Mab is added to 30 mg (4×10^8 beads) of Dynabeads. SAM-450 Dynabeads alone are used as negative control. Approximately 2×10^7 isolated MNC are re-suspended in one ml PBS with 1% human serum albumin (HSA) in a plastic tube, and immunobeads are added in a concentration of 0.5:1 to the total number of cells. After incubation of the mixture under rotation for 30 min, the cells are diluted with PBS+1% HSA, and the tube is put in a magnet holder (Dyna, Oslo, Norway). Cells reactive with the Mabs bind the beads as rosettes, and cell-bead rosettes are trapped on the wall of the test tube. The supernatant, containing unbound cells, is decanted. The remaining positive fraction in a volume of approximately 200 μl is placed on ice, and a 20 μl aliquot is examined for rosettes (Fig. 1) by microscopy, using a Zeiss Axioscope (Carl Zeiss, Jena, Germany). A sample containing at least two cells with five or more TP-3 or 9.2.27 beads attached as a rosette is regarded as positive.

Results

In this series of 41 OS-patients, the age at diagnosis ranged from 8 to 51 years, with a mean age of 16 years. DTC's were detected in 26 of the 41 patients (63%) with a mean follow up of 73 months. Among the 15 patients (seven males and eight females) that did not have micrometastases isolated from their bone-marrow aspirate – hereafter called micrometastasis negative (MM-) – none have experienced an OS-related event following adjuvant chemotherapy. Mean follow up for this cohort was 92 months. One patient died of acute leukemia nine years following the diagnosis of OS. The remaining 14 are all NED. In this MM- group, five had a primary tumor of malignancy grade 3 and 10 had OS of grade 4.

The 26 OS-patients (15 males and 11 females) in the micrometastasis positive (MM+) cohort had a significantly worse outcome (log rank $p=0.038$ – see Fig. 2). A total of seven OS-related events were observed. Despite the fact that a higher percentage of patients had grade 4 tumors (23 out of 26 tumors) in this MM+ cohort, two events were seen in patients with grade 3 OS – one of these patients is DOD and one ALVM. The remaining five events were observed in patients with grade 4 tumors; three are DOD, one ALVM and one in CR2 following a local relapse that was treated by amputation. The median follow up among MM+patients was 69 months.

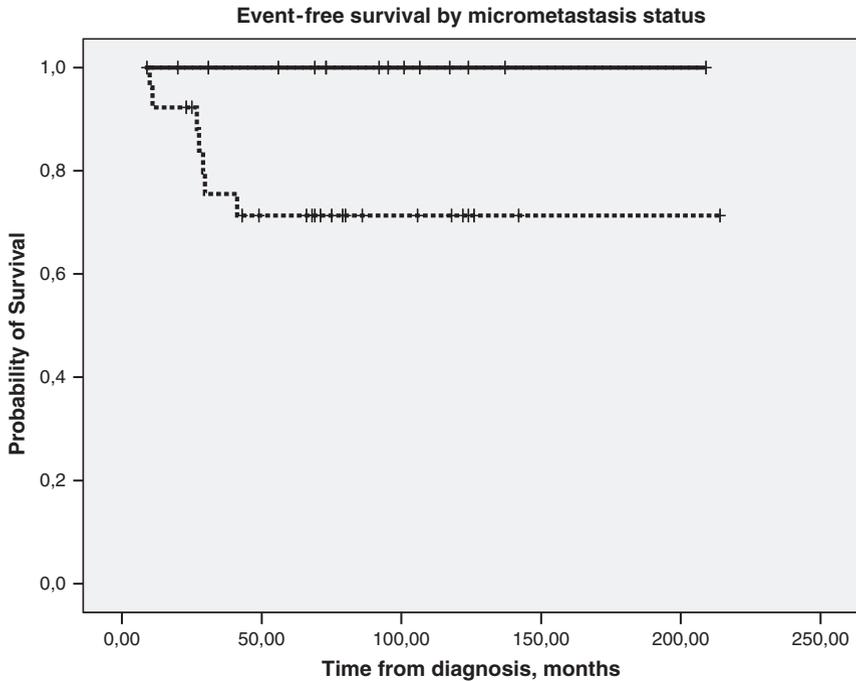


Fig. 2 Survival of 41 osteosarcoma patients by micrometastasis status, 15 micrometastasis negative patients: *whole line*. 26 micrometastasis positive patients: *dotted line*. Log rank $p=0.038$

Discussion and Future Perspectives

Experience on the prognostic impact of MM in primary bone sarcoma is so far sparse, but it has also been reported in Ewing sarcoma and other pediatric sarcomas.³¹⁻³³

Recently, improved methods enabling molecular characterization of MM by recombinant DNA-technology has been reported.³⁴ This should allow the definition of novel targets for therapy.^{16,35-39} Ideally, an adjuvant therapy should be tailored and based on properties of the MM, not of the primary tumor.

Summary

In conclusion, a very high fraction of classical OS patients had malignant cells in BM at primary diagnosis, and a significant correlation between the presence of DTCs and disease progression was found. The data demonstrate the clinical potential of this immunomagnetic method. Attempts to subgroup OS-patients for more individualized treatment based on the presence of MM cells should be studied in a

larger cohort of patients. Molecular characterization of isolated MM could identify cellular pathways as a basis for targeted adjuvant therapies.

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