NOWOTWORY Journal of Oncology • 2004 • volume 54
Number 4 • 347–353

Ki-67 expression in soft tissue sarcomas in children

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Introduction. Expression of the Ki-67 antigen is one of the markers of cell proliferation. The proliferation index has been successfully used as a prognostic marker in many malignant tumours. The aim of this study was to establish the prognostic relevance of Ki-67 antigen expression in soft tissue sarcomas in children and to compare Ki-67 expression with some selected clinical parameters.

Material and methods. 113 children (65 boys and 48 girls) aged between 1 and 217 months with diagnosed soft tissue sarcomas entered the study. Four patients were at stage I, 12 at stage II, 63 at stage III, and 34 patients, at the time of diagnosis, presented distant metastases. The activity of Ki-67 antigen was evaluated in paraffin-embedded tissue samples of tumours using the DAKO kit. The examination of Ki-67 expression was performed on Olympus BX 50 microscope connected to PC computer (equipped with Pentium 130 MHz and graphic card: Frame Grabber) and graphic MultiScan 5.10 software, Computer Scanning System. The Ki-67 expression was assessed in 500 cell of each case in several randomly chosen visual fields and the percentage of Ki-67 positive cells was calculated.

Results. The mean proliferation index in the analysed group was 18.5%. The highest average of Ki-67 expression was noted in alveolar rhabdomyosarcoma. The high activity of the Ki-67 antigen was associated with a poorer response to induction chemotherapy ($p<0.05$), and with shorter event free survival (5-years EFS: 0.44 and 0.26; $p<0.05$) and overall survival (5-years OS: 0.55 and 0.31; $p=0.03$). Moreover, patients with high Ki-67 expression had a smaller chance to survive after relapse ($p=0.011$). However, no significant difference was observed between Ki-67 activity and tumour size and stage, stage of disease, infiltration of regional lymph nodes and presence of distant metastases.

Conclusion. We conclude, that Ki-67 antigen expression is a significant prognostic marker in soft tissue sarcomas in children and that the evaluation of this parameter could be helpful in precise pre-treatment stratification and may thus optimize the efficacy of the therapeutic modalities.

Ekspresja antygenu Ki-67 w mięsakach tkanek miękkich u dzieci

Wstęp. Ekspresja antygenu Ki-67 jest uznawana za jeden z markerów aktywności proliferacyjnej komórek. Indeks proliferacyjny okazał się skutecznym czynnikiem rokowniczym w wielu nowotworach. Celem obecnej pracy było zbadanie ekspresji antygenu Ki-67 w komórkach mięsaków tkanek miękkich u dzieci i ocena wpływu tego parametru na przeżycie pacjentów, a także porównanie ekspresji antygenu Ki-67 z wybranymi parametrami klinicznymi.

Materiał i metoda. Badaniem objęto 113 dzieci (65 chłopców i 48 dziewczynek) w wieku od 1 do 217 miesięcy z rozpoznanym mięsakiem tkanek miękkich. Po pierwotnym zabiegu chirurgicznym w I stadium klinicznym choroby znajdowało się 4 pacjentów, w stadium II – 12, w stadium III – 63, natomiast postać rozsianą choroby prezentowało 34 dzieci. Aktywność antygenu Ki-67 oznaczono w utrwalonych w parafinie wycinkach tkanki nowotworowej, uzyskanych w trakcie pierwotnego zabiegu chirurgicznego, posługując się zestawem firmy Dako. Do oceny ekspresji antygenu Ki-67 używano mikroskopu świetlnego firmy Olympus BX 50 z torem wizyjnym i komputera Pentium 130 z kartą graficzną Frame Grabber oraz programu komputerowej analizy obrazu Multiscan 5.10. Obecność antygenu Ki-67 badano w 500 komórkach każdego preparatu w kilku losowo wybranych polach widzenia, obliczając odsetek komórek Ki-67 dodatnich.

Wyniki. Średni indeks proliferacyjny Ki-67 dla całej grupy wyniósł 18,5%. Najwyższe średnie wartości ekspresji antygenu Ki–67 stwierdzono w podtypie pęcherzykowym mięsaka prążkowanokomórkowego. Wysoka aktywność proliferacyjna wiązała się z gorszą odpowiedzią guza na chemioterapię wstępną ($p<0,05$), a także z krótszym czasem przeżycia wolnego od zdarzeń

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(5-letnie EFS: 0,44 i 0,26; p<0,05) oraz przeżycia całkowitego (5-letnie OS: 0,55 i 0,31; p=0,03). Wykazano ponadto, że w grupie osób z wysoką ekspresją antygenu Ki-67 istotnie mniejsze są szanse przeżycia po wznowie choroby (p=0,011). Nie stwierdzono natomiast istotnych zależności pomiędzy indeksem Ki-67 a wielkością i sposobem rozprzestrzeniania się guza, stadium choroby, zajęciem regionalnych węzłów chłonnych oraz obecnością przerzutów.
Wnioski. Na podstawie uzyskanych wyników można stwierdzić, że ekspresja antygenu Ki-67 jest istotnym czynnikiem rokowniczym w mięsakach tkanek miękkich u dzieci, a oznaczenie tego parametru pomoże uzupełnić stratyfikację pacjentów przed rozpoczęciem leczenia przeciwnowotworowego i tym samym zwiększyć skuteczność stosowanych metod terapeutycznych.

Key words: Ki-67 antigen, soft tissue sarcomas, prognosis
Słowa kluczowe: antygen Ki-67, mięsaki tkanek miękkich, rokowanie

Introduction

Soft tissue sarcomas form a heterogenous group of malignant tumours originating from embryonal mesenchymal and neuroectodermal tissue. In Poland they account about 7% of all childhood malignancies and are the fifth most common type of tumour (after leukaemias, tumours of the CNS, lymphomas and neuroblastomas) diagnosed below 16 years of age [1]. Despite a number of common biological and clinical features (including the response to anti-tumour therapy) soft tissue sarcomas vary histologically, thus causing problems in patient stratification and in the choice of the best treatment method. The currently used clinical classification system, based on the histological type of the tumour, its size and localization, patient age and the presence of metastases does not allow for an adequate prediction as to the course of the disease. Therefore we ought to identify new prognostic factors, connected to the biological features of the tumour which would allow to set more precise treatment strategies individually adapted for each patient [2]. One such parameter which raises hopes as to an improvement in the stratification of our young patients is the grade of malignant cell proliferation, because in a number of tumours high activity of cell proliferation correlates with increased malignancy [3, 4].

The level of the activity of cell proliferation may be assessed through the evaluation of the degree of the Ki-67 antigen expression, the number of nucleoli organizers and the PCNA (proliferating cell nuclear antigen). The Ki-67 antigen has been first described in the year 1983 as a nuclear protein accompanying cell proliferation. It is encoded on chromosome 10 (10q25) [5]. The full function of the Ki-67 antigen remains, as for now, unclear. It is presumed that it may participate in the regulation of transcryptic activity in the course of cell division. It is known that Ki-67 expression correlates with DNA synthesis, but it does not accompany processes of DNA repair [5]. Recent developments have rendered the evaluation of Ki-67 much easier – previously fresh tissue samples had to be frozen, at present it is possible to evaluate Ki-67 in paraffin-embedded tissue samples with the aid of MIB1-3 antibodies. This last method has also allowed to perform such analyses in historical material.

Most literature reports are concerned with Ki-67 antigen expression in soft tissue sarcomas of adult patients, in whom the high index of proliferation has been found to correlate with a high grade of malignancy and poor disease free survival (DFS) [3, 6-12]. The aim of this study is to examine Ki-67 antigen expression in tissue samples obtained from children with soft tissue sarcomas and to evaluate the influence of this parameter on tumour aggressiveness, its reaction to chemotherapy and on the outcome of treatment.

Method

Immunohistochemical evaluation of Ki-67 antigen activity

Ki-67 activity was evaluated in 4 μm thick paraffin-embedded samples placed on gelatin glass (2g $KCr(SO_4)2x12H_2O$ + 2.5g gelatine), and later de-paraffinated in xylene. The samples were then hydrated in solutions containing decreasing concentrations of ethyl alcohol and repeatedly rinsed in distilled water. The hydrated samples were then placed in pH 6.0 citrate buffer and heated in a microwave oven (95°C for 40 minutes). After cooling (20 minutes in room temperature) and rinsing twice in TBS solution the samples were incubated in 3%H_2O_2 and then rinsed in distilled water (2 x 5 minutes) and in TBS (2 x 5 minutes).

In order to prove Ki-67 expression we used rabbit serum against the human Ki-67 antigen (DAKO) in a concentration of 1:50. The samples were incubated with the serum at room temperature for 45 minutes, with simultaneous control performed on Ki-67 control slides (DACO, 1073). Excess serum was rinsed off with TBS (2 x 5 minutes). We used the Biotinylated Goat Antibody to Mouse/Rabbit Immunoglobulins (StrepABComplex/HRP Duet, Mouse/Rabbit, DAKO K0492) as the binding (intermediate) antibody, while for the indicator complex we used the StrepABComplex (StrepABComplex/HRP Duet, Mouse/Rabbit, DAKO K0492). Both reagents were incubated at room temperature for 30 minutes and then rinsed with TBS (2 x 5 minutes).

In order to visualize the reaction we used the DAB substrate (3,3-diaminobenzidine tetrachlorohydrate – DAKO DAB Tablets). 6 mg of DAB were dissolved in 0.05 TBS (pH 7.4) and 0.1 ml of H_2O_2, and the samples were incubated in this solution at room temperature for 30 minutes, and then rinsed for 15 minutes. The samples were then stained with Mayer hematoxylin, rinsed with water, covered with cover glass and Canada balsam.

In order to perform a quantitative analysis of the Ki-67 positive cells we used an Olympus BX 50 light microscope with a visual path and a Pentium 130 computer with Frame Grabber graphic card Multiscan 5.10 software for computerized picture analysis. We counted all malignant cells (both positive and negative) discernible within the visual field, and then all malignant cells showing a positive reaction as to the presence of the Ki-67 antigen. The presence of Ki-67 was evaluated in 500 cells of each sample in a few randomly chosen visual fields, and then the ratio of positive cells was calculated. Only evident brown

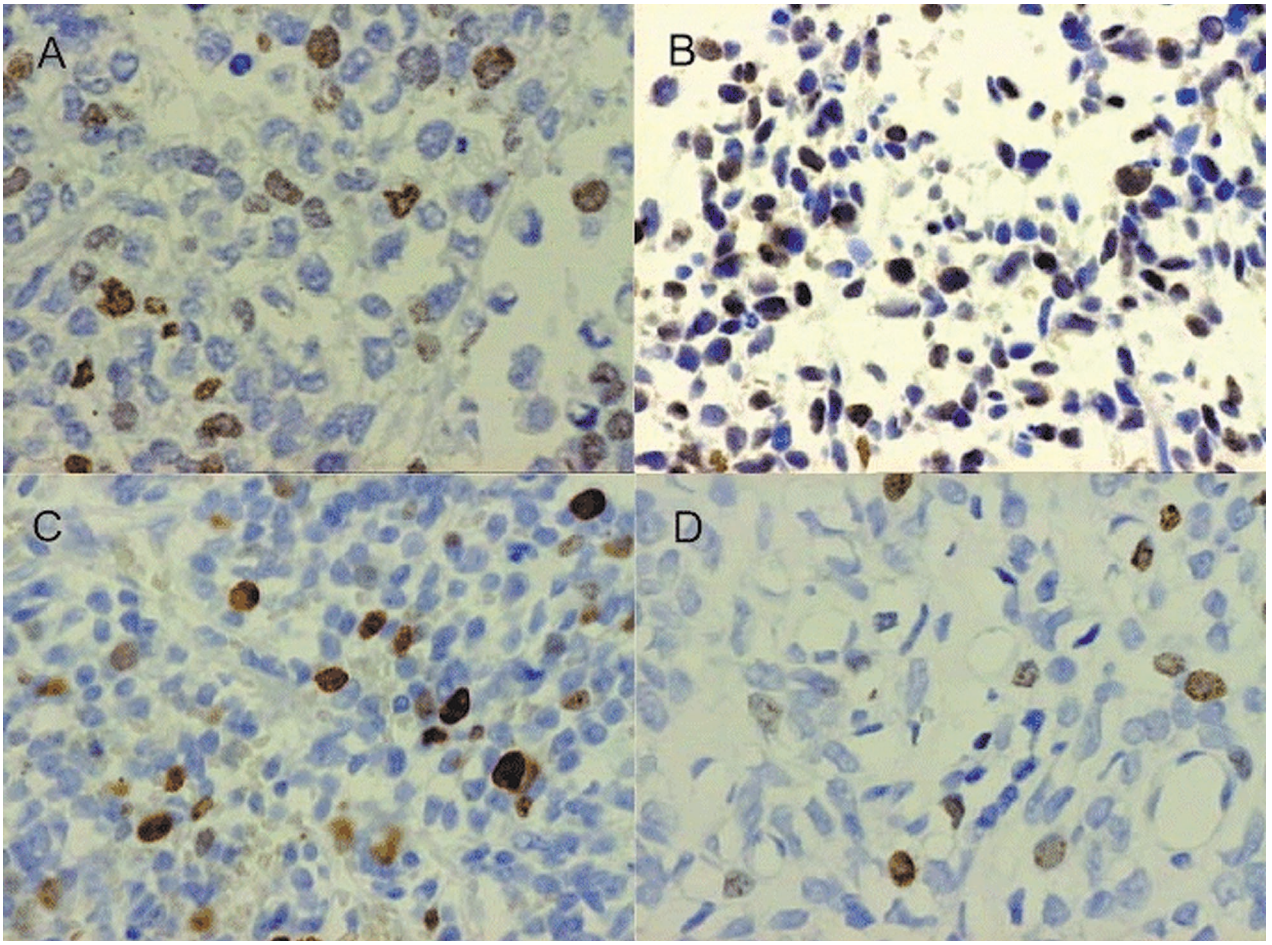

Figure 1. Microphotograph of immunohistochemically stained malignant tissue samples examined for the presence of the Ki-67 antigen. A) embryonal rhabdomyosarcoma (RME) B) alveolar rhabdomyosarcoma (RMA) C) peripheral primary neuroectodermal tumour (PNET) D) synovial sarcoma (SS)

staining of the cellular nuclei was considered as positive. In order to perform a control of the immunohistochemical analysis we performed a simultaneous reaction which repeated the entire procedure omitting only the incubation with the specific antibody. Examples of positive reactions are presented in Figure 1.

Patients

Ki-67 antigen expression had been evaluated in paraffin-embedded tissue material obtained from 113 children treated by the Polish Paediatric Solid Tumour Group. Altogether we analysed the cases of 65 boys (57.5%) and 48 girls (42.5%) aged between 1 and 217 months (mean age: 105.2 + 65.2 months, median: 102 months). In 55 cases (48.7%) we recognized the embryonal form of the rhabdomyosarcoma (RME), in 23 cases (20.4%) – the alveolar rhabdomyosarcoma (RMA), in 6 cases (5.3%) – the undifferentiated rhabdomyosarcoma (RMU). In further 18 cases (15.9%) we diagnosed peripheral primary neuroectodermal tumours (PNET), in 6 cases (5.3%) – synovial sarcoma, in 4 cases (3.5%) – extraosseous Ewing sarcoma (EES) and in 1 case (0.9%) poorly differentiated unclassified sarcoma (UKS). As to the clinical stage of the disease 4 patients (3.5%) were in stage I, 12 patients (10.6%) in stage II, 63 patients (55.8%) in stage III, while in the remaining 34 patients (30.1%) distant metastases were present at the time of diagnosis. Regional lymph node involvement (N1 acc. to the TNM classification) was observed in 26 patients (23%).

In 29 cases (25.7%) the primary tumour was limited to the organ or tissue of origin (stage T1 acc. to the TNM classification), while in 81 patients it infiltrated neighbouring organs (stage T2). In 3 cases (2.6%) it was impossible to discern the character of the primary tumour due to the lack of sufficient data. In 23 cases (20.4%) the diameter of the primary tumour did not exceed 5 cm, while in 88 cases (77.9%) it was over 5 cm. In 2 patient the size of the primary tumour was not established. At the time of analysis (May 2002) 59 patients (52.5%) were still alive, the 54 remaining patients (47.8%) having died.

Statistical analysis

Statistical analysis was performed with the aid of the Statistica®97Pl for Windows® software. In order to establish differences between patient groups we used Student's T-test (if parameter distribution was close to normal). In case of non-parametric values we used the $\chi 2$ test, and in groups with less than 5 subjects – the Fisher's exact test [13]. The probability of event-free survival (EFS) was evaluated with the aid of the Kaplan-Meier method [14] beginning with the day of therapy onset and ending with the failure day or with the day of the most recent patient observation. Failure was defined as progression, recurrence or death (regardless of cause). The probability of overall survival (OS) was calculated beginning with day 1 of treatment until the time of death or the day of the most recent patient observation. Possible differences in the calculated probability of OS and EFS were verified with the log-rank test [15], and in case of groups less numerous than 15 – with Cox's F test. The confidence level of the analysis was set at $\alpha=0.05$, significance level $p<0.05$.

Results

Due to the lack of a standard value at which Ki-67 antigen expression could be considered as high for the sake of this particular study we turned to literature reports and assumed this level to be set at 20% of cells with Ki-67 antigen expression [6, 7, 12]. Tumours in which at least 20% of cells revealed Ki-67 expression were considered as those with a high proliferative grade, while tumours in which Ki-67 expression was found in less than 20% of cells were considered to possess low proliferative activity.

Ki-67 expression was found in the case of 107 (94.7%) tumours. The mean Ki-67 proliferative index was 18.5% in the entire group (range: 0%-44.3%); for the rhabdomyosarcoma group (RMS) – 19.2% (range: 0%-44.3%); for the remaining patients (non-RMS) – 17.9% (range: 0%-40.9%). The highest mean values of Ki-67 antigen expression were found in the RMA group (27.4%), and they were significantly higher than in the RME group (15.5%; $p<0.00001$). Moreover, in case of RMA tumours with a higher proliferation grade (>20% of Ki-67 positive cells) were significantly more common than in RME (RMA < 20% and >20% – 5 and 18 patients respectively; in RME – 38 and 17 patients respectively; $p<0.001$). In the case of RMU the mean Ki-67 was 21.7%, and for synovial sarcoma – 23.9%. The lowest expression was observed in EES – reaching 5.6% and although statistical verification has shown that the mean values in this group differ significantly as compared to RMA ($p<0.001$), RME ($p<0.05$), PNET ($p<0.05$) and SS ($p=0.017$) groups, yet the very low number of patients (n = 4) calls for a very careful approach to this data.

Tumour size, clinical stage, regional lymph node involvement, the type of tumour spread and the presence of metastases did not reveal any statistical significant differences as to the grade of proliferation, both regarding the mean value of Ki-67 positive cells and regarding the frequency of tumours with a high proliferative activity. However, one cannot fail to notice the slightly higher proliferative activity in more advanced stages of the disease (stages I-III as compared to stage IV – 17.9% and 21%, respectively); in the case of primary tumours over 5 cm in size (<5 cm vs. >5 cm – 16.7% and 19.6%, respectively); in case of tumours infiltrating neighbouring organs (T1 vs. T2 – 18.5% and 19.1%, respectively) and in the case of regional lymph node involvement (N0 vs. N1 – 18.3% and 21.3%, respectively). We have also noticed that proliferative activity correlates with the reaction of the tumour to initial chemotherapy. Among the 84 patients in stage III and IV in whom it was possible to evaluate the response after the first cycle of chemotherapy we observed complete remission and good response (tumour regression >2/3 of initial size) more often in patients with Ki-67 expression <20% (25/47 patients as compared to 12/37 patients with Ki-67 expression >20%). On the other hand, tumours with partial response (regression >1/3 and < 2/3 of initial size) and no response (<1/3 regression or progression) were more frequent in the group with the >20% index (Ki-67 expression <20% and >20% – 22/47 and 25/37, respectively; $p<0.05$). Treatment failures were also more common among patients with high proliferative activity. At the closing of the analysis in the group with Ki-67 expression <20% 23 patients had died (37.1%), while in the Ki-67 >20% group there were 31 deaths (60.8%) ($p=0.012$). It is also worth noting that among the patients who had died the mean values of Ki-67 expression were significantly higher, despite the administration of oncolytics, as compared to the surviving patients (21.1% vs. 16.8%, respectively; $p=0.04$).

EFS and OS of the analysed patients are presented in Figures 2 and 3. We have shown that patients with a low proliferative activity (Ki-67 <20%) has a significantly higher likelihood of both event free survival (5 year EFS 0.44 and 0.26, respectively; $p<0.05$) and of overall survival (5 year OS 0.55 and 0.31, respectively; $p=0.03$). We have also shown, that the proliferative

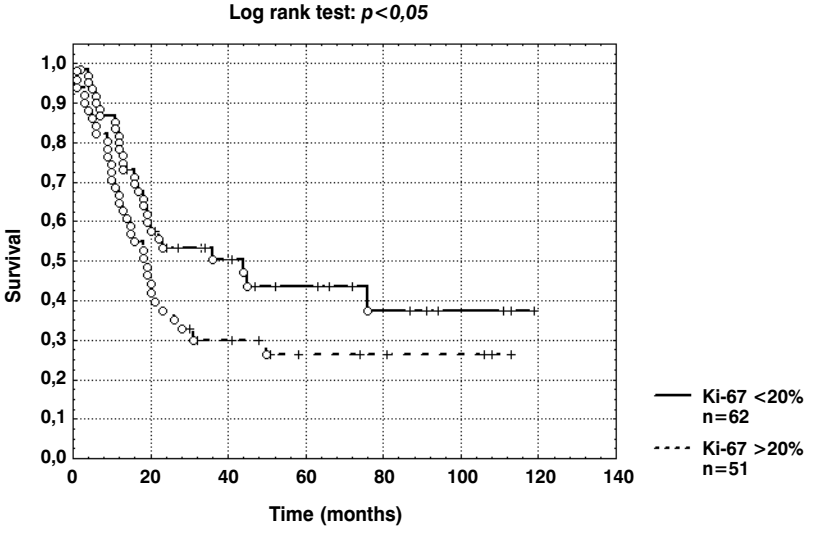

Figure 2. Event free survival (EFS) in relation to the grade of Ki-67 antigen expression evaluated in tumour cells

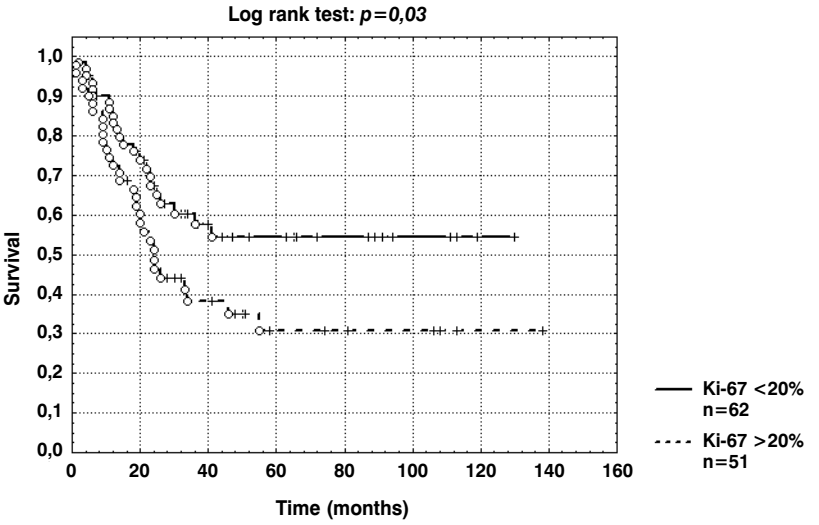

Figure 3. Overall survival (OS) in relation to the grade of Ki-67 antigen expression evaluated in tumour cells

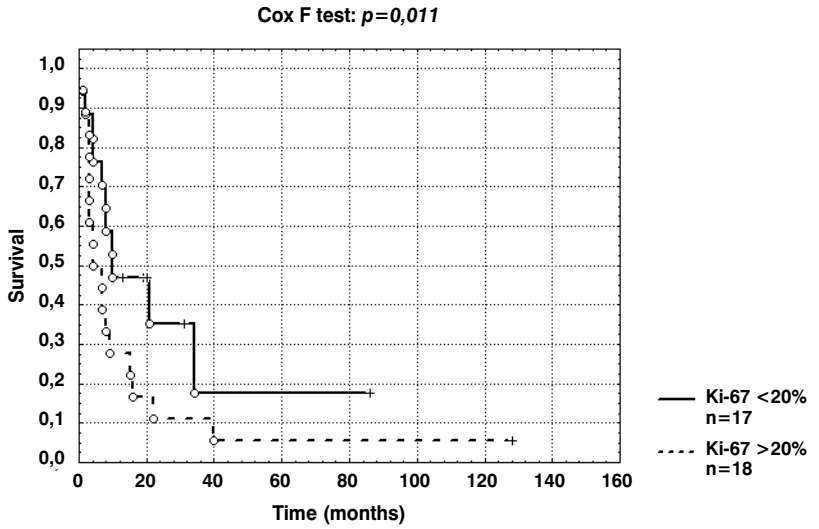

Figure 4. Patient survival after relapse in relation to the grade of Ki-67 antigen expression evaluated in tumour cells

activity is also a factor influencing prognosis among patients with recurrent disease (Figure 4). Patients with the Ki-67 index of <20% stand a better chance for longer survival and next disease remission as compared to patients with the Ki-67 index of >20% (the ratio of patients still alive after 60 months of follow up from the time of recurrence being 0.18 vs 0.07, respectively; p=0.011).

Discussion

The Ki-67 antigen is a commonly recognized marker of cell proliferation. Literature reports concerning the activity of Ki-67 in soft tissue sarcomas are few, and they are mainly devoted to adult patients [6-12]. In those patients the high proliferative index distinctly correlates with low EFS, while the level of Ki-67 antigen expression correlates with the degree of histological malignancy of the tumour [3, 9, 10]. It has also been shown, that a high Ki-67 index (>20%) correlates with a higher risk of developing distant metastases [8, 12]. However, due to a number of specific characteristics of childhood sarcomas, as compared to those in adult patients, the data obtained from adults cannot be directly applied in the clinical practice concerning patients below 18 years of age. There is, as for now, no extensive literature report concerning Ki-67 antigen expression in childhood sarcomas. We have found only one report on this issue concerning 25 rhabdomyosarcoma patients, whose median age was 10.9 years [16]. For that reason we have attempted to perform an evaluation of this parameter in the stratification of soft tissue sarcomas of children and adolescents.

The analysis of our material has shown, that evaluation of the Ki-67 antigen expression in malignant cells may be useful for the evaluation of risk factors among children with soft tissue sarcomas. EFS and OS were significantly lower in the group of patients with a high proliferative index, as compared to the group of patients with a low proliferative index. It is worth noticing,

that the proliferative activity of the tumour correlated not only with the final treatment outcome, but it also allowed to predict, at least partially, the response of the tumour to initial chemotherapy. Among patients with a high Ki-67 index satisfactory tumour regression (>2/3 of initial size) was significantly less common after the first cycle of chemotherapy.

An analysis of Ki-67 antigen expression has also allowed to evaluate the prognosis of patients with recurrence. A higher proliferative index found in the material obtained before the onset of treatment correlated with earlier death after recurrence. This finding is all the more important because in the case of malignancies with a very high risk of failure the presently-used stratification systems do not allow to identify subjects with poorer or better prognosis [2,12,17]. In the course of our study we have not managed to confirm the role of the Ki-67 antigen in the development of distant metastases. It may be explained by the fact that the biology of childhood sarcomas is somewhat different. On the other hand it cannot be overruled that our group of patient with metastases was not very numerous (n=34) and therefore some statistically significant differences may have failed to appear. Certainly further studies are necessary.

The evaluation of the Ki-67 antigen expression may also be useful in the histopathological diffrentiation of soft tissue sarcomas. Basing on our material we may conclude that the grade of Ki-67 expression correlates with the histopathological type of the tumour, which may render histological diagnosis easier.

The role of the Ki-67 antigen in the oncogenesis of sarcomas remain unclear. Initial reports suggest the participation of this protein in the complex interactions occurring during the cellular cycle. It has been observed, that in cases of Ewing's sarcoma the level of the Ki-67 antigen correlates with the expression of the c-myc protooncogene [18]. This oncogene has been found to amplify in a number of cancers, neuroblastoma-type tumours and mesenchymal tumours [19]. It has also been established that a high Ki-67 index accompanies the nuclear overexpression of the p53 protein, which is considered to be a sign of mutation within the TP53 suppressor gene [7]. Also, in the case of synovial sarcoma the high level of Ki-67 expression correlates with the presence of a specific gene fusion SYT-SSX1 characteristic for this very malignancy [20]. The role of this mutation in the process of oncogenesis is not yet clear, however it is suggested that its presence is somehow related with the significantly worse prognosis of synovial sarcoma patients [20]. Further studies on the role of the Ki-67 protein in the cellular cycle and in the process of oncogenesis are necessary.

To summarise we may state, that Ki-67 activity may be a useful marker helpful in the process of clinical examinations designed to assign the patient to a certain risk group. The analysis of Ki-67 activity is simple to perform and inexpensive. It may be performed both from fresh tissue samples and from paraffin-embedded samples, thus allowing to perform retrospect analyses.

Acknowledgements

The authors wish to thank the following persons who have been kind enough to submit tissue samples and clinical data of patients: Professor Anna Balcerska (Dept. of Paediatrics, Haematology, Oncology and Endocrinology, Gdańsk), Asst. Professor Walentyna Balwierz (Dept. of Paediatric Haematology, The Polish-American Institute of Paediatrics, Kraków), Professor Jerzy Bodalski (Dept. of Children's Diseases, Łódź), Elżbieta Drożyńska MD (Dept. of Paediatrics, Haematology, Oncology and Endocrinology, Gdańsk), Agnieszka Dłużniewska MD (Dept. of Paediatric Haematology, The Polish-American Institute of Paediatrics, Kraków), Jan Domaniewski MD (Dept. of Pathomorphology, Bydgoszcz), Krzysztof Kątski MD (Dept. of Paediatric Haematology and Oncology, Lublin), Jacek Kijowski MD (Dept. of Paediatric Surgical Oncology, Institute of the Mother and Child, Warszawa), Asst. Professor. Józef Kobos (Laboratory of Pathomorphological Diagnostics, PSK 4, Łódź), Jadwiga Koronkiewicz MD (Dept. of Pathomorphology, Voivodeship Hospital, Bydgoszcz), Professor Jerzy Kowalczyk (Dept. of Paediatric Haematology and Oncology, Lublin), Andrzej Kurylak MD (Dept. of Paediatrics, Haematology and Oncology, Bydgoszcz), Professor Maria Liebhart (Dept. of Pathomorphology, Institute of the Mother and Child, Warszawa), Jadwiga Małdyk MD (Dept. of Pathomorphology of Developmental Age, Warszawa), Witold Mieżyński MD (Dept. of Pathomorphology, Collegium Medicum, Kraków), Professor Danuta Perek (Dept. of Paediatric Oncology, Child's Health Centre, Warszawa), Professor Roma Rokicka-Milewska (Dept. of Haematology and Coagulopathies, Warszawa), Professor Andrzej Roszkiewicz (Dept. of Pathomorphology, Gdańsk), Magda Rychłowska MD (Dept. of Paediatric Surgical Oncology, Institute of the Mother and Child, Warszawa), Danuta Skomra MD (Dept. of Pathomorphology, Lublin), Dariusz Stencel MD (Dept. of Paediatric Haematology and Oncology, Institute of Paediatrics, Poznań), Beata Szewczyk MD (Dept. of Children's Diseases, Łódź), Barbara Sopyło MD (Dept. of Haematology and Coagulopathies, Warszawa), Ewa Trejster (Laboratory of Clinical Pathomorphology, Institute of Paediatrics, Poznań), Professor Jacek Wachowiak (Dept. of Paediatric Haematology and Oncology, Institute of Paediatrics, Poznań), Maria Wieczorek MD (The Chorzów Centre of Paediatrics and Rehabilitation, Chorzów), Professor Wojciech Woźniak (Dept. of Paediatric Surgical Oncology Institute of the Mother and Child, Warszawa), Professor Mariusz Wysocki (Dept. of Paediatrics, Haematology and Oncology, Bydgoszcz).

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Paper received: 3 November 2003
Accepted: 6 January 2004