Image analysis derived ploidy and proliferation indices in soft tissue sarcomas: Comparison with clinical outcome

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Abstract

Aims—To compare prognostic information obtained by image analysis cytometry of paraffin wax embedded soft tissue sarcomas with conventional assessment.

Methods—A CAS 200 image analyser was used to determine DNA content of Feulgen stained cytology preparations and tissue sections and to quantify immunostaining by Ki67 and PC10 antibodies. A mitotic count in 50 high power fields was undertaken and histological grade assigned by the Trojani system. Clinical details including follow up and outcome were obtained by case note review. The Kruskal-Wallis one way analysis test, Spearman ρ significance test, Kaplan-Meier method, and log-rank test were applied in statistical analysis.

Results—Ploidy status, DNA index, 2·5c exceeding rate, 5c exceeding rate, mitotic count and Trojani grade all correlated significantly with clinical outcome. The relation between Ki67 index and outcome did not reach significance. The PC10 index and outcome were not related. Only 2·5c exceeding rate, 5c exceeding rate, and mitotic count correlated significantly with Trojani grade.

Conclusions—DNA content determination of soft tissue sarcomas by image analysis provides quantifiable information of benefit in prediction of outcome. Larger series are required to determine the independent value of ploidy. In this study quantification of anti-Ki67 and anti-PC10 immunostaining was not of prognostic benefit by contrast with mitotic count and Trojani grade.

(J Clin Pathol 1994;47:443-447)

Soft tissue sarcomas are often associated with poor patient survival. Conventional assessment of prognosis includes evaluation of histological type, grade, anatomical site, and stage. Histological type alone is not usually a prognostic indicator whereas grade is more predictive.¹ The most reproducible soft tissue sarcoma grading system to date is the Trojani system, a scoring system that assesses degree of differentiation, extent of necrosis, and mitotic activity.¹ Although of recognised value as an indicator of prognosis there are numerous instances where Trojani grade and outcome are not in concordance.² The work of Enneking³ has led to improved clinicopatho-

logical staging of soft tissue tumours although some of the staging criteria are difficult to apply.

There is thus a need to identify additional prognostic indicators in this discipline. Image analysis cytometry offers a means of measuring DNA content (ploidy) while preserving tissue architecture and cytological detail. Ploidy determination by image analysis is a significant prognostic indicator in many malignancies.⁴⁻⁸ Similarly, immunostaining of cell cycle related antigens, for example Ki67 and PC10, has been widely used to predict survival.⁹⁻¹⁰ Microwave retrieval¹¹ allows application of Ki67 antibody to retrospective paraffin embedded material. This study was undertaken to determine if ploidy and proliferation data obtained by image analysis of paraffin wax embedded soft tissue sarcomas correlate with patient survival and to compare these data with conventional assessment of Trojani grade and mitotic count.

Methods

Thirty three soft tissue sarcomas were selected from the files of the Scottish Bone Tumour Registry and the Pathology Department. The sample was chosen to examine a range of histological types and a range of grades. Case note review was undertaken and histological diagnosis, site, follow up, treatment, and survival was extracted. All haematoxylin and eosin stained slides for each case were reviewed and one tissue block was selected from the most poorly differentiated area. Only prechemotherapy or preradiotherapy blocks were used. Sections (3 μ m) for proliferation analysis by immunostaining were mounted on poly-l-lysine coated slides, 3, 5, 7, 9, 11, and 15 μ m sections for ploidy analysis were mounted on conventional slides and a 50 μ m section for disaggregation placed in a glass universal container. All sections were dewaxed in xylene and hydrated in graded alcohols.

The 50 μ m sections were disaggregated by a modification of the standard method of Hedley *et al.*¹² The sections were incubated for 120 minutes at 37°C in 2 ml of 0·9% pepsin in normal buffered saline adjusted to pH 1·5 with 2N HCl. The resulting suspension was centrifuged for three minutes and the pellet resuspended in buffered saline and cytocentrifuged on to glass slides in a Shadow Cytospin 2 at 650 rpm for five minutes.

The cytospin and histology sections for ploidy analysis were hydrolysed in 5N HCl for

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Accepted for publication
25 November 1993

60 minutes and transferred to Feulgen stain solution (Becton-Dickinson, Oxford, UK) for 60 minutes. After three rinses in rinse reagent (Becton-Dickinson, Oxford, UK) the slides were washed in deionised water, immersed in acid alcohol for five minutes, dehydrated, cleared in xylene, and coverslips were added. A control slide imprinted with rat hepatocytes of known DNA content was added to each batch to calibrate the image analyser and ensure adequate staining.

The 3 µm cytospin slide section and the thickest section from the remainder (5–15 µm) from which sufficient nuclei could be counted were analysed for each case with a CAS 200 Image Analyser (Becton-Dickinson, Oxford, UK). A minimum of 300 tumour cells and 50 diploid control cells (lymphocytes, polymorphs, or connective tissue cells) were analysed and the data presented as a DNA histogram. In tissue sections control and tumour cell spindle shaped nuclei were only measured in the longitudinal plane.

Ploidy was assigned by combined interpretation of cytospin and tissue section histograms. An average DNA index of the main histogram peak for each case was calculated. A DNA index of 0.9–1.1 was accepted as diploid, <0.9 or 1.11–1.30 as near diploid aneuploid, and >1.3 as aneuploid. The 2.5c exceeding rate and 5c exceeding rate are the proportions of tumour nuclei with a DNA content of more than 2.5 and 5 times haploid respectively. Average 2.5c and 5c exceeding rates were calculated for each case and expressed as a percentage.

Tissue sections for Ki67 immunostaining were microwaved twice for five minutes in

preboiled 10 mM citrate buffer adjusted to pH 6.0 with 2N sodium hydroxide. Primary Ki67 monoclonal antibody (Dakopatts, High Wycombe, UK) was applied at 1:25 dilution, incubated for 120 minutes at room temperature, and binding detected by the avidin-biotin method. Primary PC10 monoclonal antibody was applied at a one in 200 dilution and incubated for 16 hours at 4°C and immunostaining was detected by the same method. All immunostained sections were counterstained with methyl green. Small intestine, lymph node, proliferative endometrium, myometrium and skin sections were included in each immunostaining batch as controls and a negative control (same protocol without primary antibody) was included for each case.

Immunostaining was quantified by measuring a minimum of 20 random fields or 1000 nuclei with CAS 200 Quantitative Proliferation Index software. Mitotic figures in 50 high power fields ($\times 400$ magnification, field area = 0.2 mm²) were counted. Trojani grade was assigned using all pretreatment haematoxylin and eosin sections in view of the need to assess the extent of necrosis.

The significance of any correlation between the continuous variables (DNA index, 2.5c exceeding rate, 5c exceeding rate, Ki67 index, PC10 index, and mitotic count) and Trojani grade was calculated with the Kruskal-Wallis one way analysis test, and the relation between ploidy assignment and grade by the Spearman ρ test. The 2.5c exceeding rate, 5c exceeding rate, mitotic count, Ki67 index, and PC10 index were each separated into high and low groups by the median. Survival curves were used as the measure of clinical

Ploidy and proliferation indices in soft tissue sarcomas

Classification	Diagnosis	Follow up (months)	Trojani grade	Clinical status	Ploidy	DNA index	2.5c (%)	5c (%)	Mitotic count	Ki67 index	PC10 index
1	Fibrosarcoma	60	II	DOD	A	1.35	50.4	4.1	30	0.78	24.4
2	Myxoid fibrosarcoma	36	I	NED	A	1.50	74.5	6.6	0	3.23	21.2
3	Malignant fibrous histiocytoma	21	II	EOD	A	2.26	41.3	12.3	3	1.38	8.2
4	Myxoid liposarcoma	131	I	NED	D	1.04	14.3	1.2	2	2.55	9.2
5	Myxoid liposarcoma	120	II	MD	NDA	1.28	50.1	1.2	1	0	23.4
6	Myxoid liposarcoma	50	II	DOD	A	2.41	50	11.5	0	1.28	57.5
7	Myxoid liposarcoma	31	I	DOD	NDA	1.15	4.35	0	0	6.24	1.9
8	Round cell liposarcoma	12	I	DOD	NDA	1.10	4.3	0	0	1.29	0
9	Round cell liposarcoma	6	II	MD	NDA	1.22	27.2	0.8	0	1.32	1.2
10	Leiomyosarcoma	13	III	MD	A	2.74	68.3	45.5	30	0.78	5.1
11	Leiomyosarcoma	14	II	NED	A	1.72	52.2	19.4	11	8.96	2.7
12	Alveolar rhabdomyosarcoma	48	III	DOD	A	1.41	86	8.6	89	2.07	24.5
13	Haemangiopericytoma	38	I	EOD	D	1.08	21.8	0.3	9	3.37	10.2
14	Haemangiopericytoma	33	I	NED	D	1.04	22.4	2.8	15	0.93	3.2
15	Synovial sarcoma	60	II	EOD	NDA	1.13	21	0.6	36	0	16.4
16	Synovial sarcoma	98	I	NED	NDA	0.82	7	0	5	1.17	20.9
17	Synovial sarcoma	12	I	EOD	D	0.99	10.4	0.2	12	2.83	1.3
18	Malignant schwannoma	44	III	NED	NDA	1.17	32.8	11.6	128	0	0
19	Malignant schwannoma	17	II	DOD	A	2.75	88.7	52	0	0.5	33.8
20	Malignant schwannoma	55	I	EOD	D	0.96	8.7	0	6	0	1.6
21	Malignant mesenchymoma	36	II	DOD	A	3.13	99	82	85	1.34	51.8
22	Epithelioid sarcoma	75	II	MD	NDA	0.96	4.9	0.4	4	1.78	14.2
23	Epithelioid sarcoma	133	II	NED	NDA	1.11	37.7	4.8	6	0	48.4
24	Epithelioid sarcoma	72	II	NED	D	1.09	27.2	1.8	8	0	25.8
25	Epithelioid sarcoma	131	II	NED	NDA	1.13	70.6	11.6	3	0	29.4
26	Epithelioid sarcoma	14	I	MD	A	1.72	63.8	11.2	7	0	3.6
27	Soft tissue sarcoma	21	III	DOD	A	1.63	73	12.2	126	0.31	2.4
28	Soft tissue sarcoma	48	II	NED	D	0.98	8.7	0	5	0	3.1
29	Soft tissue sarcoma	24	III	DOD	D	0.93	41	5.3	81	0.93	47.9
30	Soft tissue sarcoma	6	I	NED	NDA	1.24	41.6	3.8	1	0	4
31	Soft tissue sarcoma	27	III	DOD	A	2.86	61.7	39.5	195	2.46	43.9
32	Soft tissue sarcoma	6	III	NED	NDA	1.28	49.3	2.4	8	0	4.1
33	Alveolar soft part sarcoma	121	I	NED	NDA	1.19	37	1.5	0	0.91	7.8

NED = no evidence of disease; EOD = evidence of disease; MD = metastatic disease; DOD = died of disease; D = diploid; NDA = near diploid aneuploid; A = aneuploid.

Figure 1 Relation between DNA index and survival.

Figure 2 Relation between Ki67 index and survival.

outcome and plotted by the Kaplan-Meier method and significance was calculated by the log-rank test.

Results

The table presents the data. When compared with outcome, aneuploidy ($p = 0.007$), high DNA index (>1.3 , $p = 0.05$; fig 1), high 2·5c exceeding rate ($>37.7\%$, $p = 0.0035$), and high 5c exceeding rate ($>3.8\%$, $p = 0.003$) were each associated with significantly diminished survival. Similarly high mitotic count ($>10/50$ at high power fields, $p = 0.035$) and Trojani grade III ($p = 0.021$) were associated with poor survival. There was a trend when Ki67 index was plotted against outcome (fig 2) but significance was not attained ($p = 0.88$).

Mitotic count ($p = 0.001$, fig 3), 2·5c exceeding rate ($p = 0.03$), and 5c exceeding rate ($p = 0.003$) correlated significantly with Trojani grade whereas ploidy assignment ($p = 0.131$), DNA index ($p = 0.159$), Ki67 index ($p = 0.574$), and PC10 index ($p = 0.108$) did not.

PLOIDY ANALYSIS

Adequate Feulgen staining of calibration slides was achieved in each batch. Insufficient control nuclei were identified in one cytopsin

Figure 3 Relation between Trojani grade and mitotic count.

preparation where the average human cellular DNA content of 7.18 pg was used to position diploidy in relation to the histogram. In the remaining cytopins lymphocyte nuclei were used as controls in 25 cases and polymorphonuclear granulocytes in seven. No tissue section histograms were generated in six cases due to excess nuclear overlap. In these cases the cytopsin alone was utilised. Only the 3 μ tissue section was suitable for ploidy analysis in a further six cases. Benign non-endothelial spindle cells were used as controls in most (65%) tissue sections and lymphocytes were used as control cells in the remainder.

Average coefficient of variation of the main peak ranged from 2.41% to 28.4% (mean 9.3%). This value was diminished in cytopins (mean 6.9%) indicating generally narrower peaks. In one case a diploid histogram was obtained by cytopsin analysis of presumed tumour cells whereas tissue section histograms were of a clearly aneuploid tumour. A total of three hypodiploid near diploid aneuploid (DNA index <0.9) histograms were generated.

PROLIFERATION ANALYSIS

Both anti-Ki67 and anti-PC10 immunostaining were confined to nuclei, and control tissues revealed staining patterns expected for proliferation markers: the proliferative compartments of endometrium, intestinal crypts, and lymph nodes were positively stained whereas myometrium and gastrointestinal stromal cells were negative. Negative controls were not immunostained. In general anti-Ki67 and anti-PC10 immunostaining was uniform. There was no anti-Ki67 immunostaining in 11 cases and anti-PC10 was negative in two. Indices obtained for PC10 (mean 16.7%) were generally higher than those for Ki67 (mean 1.58%). There was no statistical correlation between the proliferation indices (Ki67 index, PC10 index, and mitotic count).

Discussion

Ploidy status and related indices (DNA index, 2.5c exceeding rate, and 5c exceeding rate) correlated significantly with survival. These results indicate that image analysis ploidy assessment is a useful prognostic indicator in soft tissue sarcomas. The value of 2.5c and 5c exceeding rates has been recognised before.¹³ Interestingly, these indices also correlated with Trojani grade whereas ploidy assignment and DNA index did not. There are conflicting results with regard to the relation between ploidy and Trojani grade in previous studies.^{14 15} It is our opinion that it is more important that a potential indicator of prognosis correlate with survival rather than grade. Larger series are required to assess independently the value of ploidy in this discipline by multivariate analysis and to assess ploidy of individual histological types of soft tissue sarcoma.

The acceptable range of diploid by image analysis is debatable. Some authors accept up to a DNA index of 1.25 as diploid whereas others recognise near diploid aneuploidy.⁶

Hypodiploid histograms were generated in three instances in the current study. The possibility that tumour cells can lose DNA (hypodiploidy) during replication—for example, by deletions—leads to theoretical misgivings regarding flow cytometry where the first histogram peak is usually regarded as diploid without any means of excluding hypodiploidy.

In some previous studies 100–200 tumour cell nuclei were measured.^{13 14 16} Recently it has been recognised that for statistical purposes measurement of 300 nuclei is more accurate.¹⁷ Hence 300 tumour cells and 50 control cells were measured in each sample in this study. In most previous series lymphocytes have been used to position diploidy and lymphocytes are recognised as adequate control cells in cytology preparations.¹³ In tissue sections the small nuclear area of a lymphocyte can lead to setting of an incorrect low diploid DNA content value. Similarly, the spiral configuration of endothelial cells in tissue sections can lead to under-representation of tissue section diploidy.¹⁶ Both lymphocytes and endothelial cells were avoided as controls in tissue sections in this study.

Preservation of tissue architecture and ease of preparation are the advantages of ploidy assessment from tissue sections. There are, however, theoretical drawbacks in that the number of incomplete partially sectioned nuclei increases with decreasing section thickness. There was broad agreement between cytology preparation data and tissue section data in this study and in one case tissue section analysis was critical to accurate ploidy assignment. No ploidy data were obtained on tissue sections due to excess nuclear overlap in 18% of the cases. We believe that tissue section ploidy analysis should only be used in combination with cytology preparation analysis, which is recognised as the “gold standard” because most nuclei are intact.

The proliferation analysis results are less conclusive. There was a recognisable trend between high Ki67 index and poor outcome but significance was not attained. The protocol for application of Ki67 antibody to paraffin embedded material has only recently become available¹¹ and presumably the value of this antibody as a prognostic indicator will soon be elucidated. There are theoretical misgivings about the suitability of PC10 antibody as a proliferation marker and prognostic indicator due to the role of the antigen in DNA repair, its long half life, its expression in non-cycling cells, and its inducibility by growth factors.¹⁰ The PC10 index was of no value in this study.

Mitotic count is often used as a routine prognostic indicator, is of recognised value in soft tissue sarcomas, and is incorporated in the Trojani grading system. It is not surprising that mitotic count and Trojani grade correlated significantly with each other and with survival.

In conclusion, assessment of ploidy by image analysis is of value in the prediction of likely outcome in a patient with a soft tissue sarcoma established by biopsy. Similarly, conventional assessment of Trojani grade and

mitotic count is also of proved utility. Ki67 index requires further evaluation in this field, whereas PC10 index is unlikely to be useful.

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