Targeted Therapy for Ewing's Sarcoma: Significance of Heterogeneity

A.C.M. VAN DE LUIJTGAARDEN[1], W.T.A. VAN DER GRAAF[1], I. OTTE-HÖLLER[2], H.W.B. SCHREUDER[3], Y.M.H. VERSLEIJEN-JONKERS[1] and P.J. SLOOTWEG[2]

Departments of [1]Medical Oncology, [2]Pathology and [3]Orthopaedic Surgery, Radboud University Nijmegen Medical Centre, P.O. Box 9101, 6500 HB Nijmegen, the Netherlands

Abstract. *Background: Survival in Ewing's sarcoma (ES) is limited. Experience with insulin-like growth factor targeting drugs, which require specific molecular tumour alterations, herald a major breakthrough. We screened for tumour heterogeneity within patients by DNA quantification. Materials and Methods: DNA image cytometry (IC) was performed on 41 samples from 21 patients, evaluating if ploidy state remained constant over time and between different lesions within patients and the prognostic value of ploidy was assessed. Results: DNA content varied over time and different ploidy states were found to coexist at a single timepoint. Non-diploid DNA content was associated with shorter overall survival (median, 19 vs. 84 months, p=0.047). Conclusion: We encountered a change and heterogeneity of ploidy state. This implies that screening for targets on a single tumour sample is insufficient and may lead to under- or overtreatment. The fact that non-diploid DNA content was associated with an adverse outcome confirms that this technique discriminates biologically different tumour clones.*

Ewing's sarcoma (ES) is the second most common bone cancer in children and adolescents. The addition of chemotherapy to local therapy has greatly improved prognosis for patients with localized disease, from 10% to approximately 70% survival. In the 25% of patients with metastatic disease however, survival is still limited to 20% (1). Furthermore, the aggressive therapeutic approach makes survivors of bone cancer particularly at risk for late treatment-related adverse events (2). The search for new therapeutic options is therefore still ongoing. Similar to cancer research as a whole, this search is focused on targeted therapies that interfere with cancer-specific molecules rather than drugs that simply affect all rapidly dividing cells. For the first time in decades, there is reason for optimism regarding a new class of drugs. In cohorts of heavily pretreated ES patients, several complete responses have been observed at the cost of only mild toxicity with insulin-like growth factor (IGF) pathway interference-based drugs (3, 4). As a logical next step, efforts are being made to identify the subgroup of patients who will most likely benefit from these agents. Pioneering authors suggest that expression levels of IGF-1 receptor (5-8), IGF-1 (6) and IGF-2 (6) predict response to IGF-targeted therapies, whilst IGF-binding protein 3 expression (6) indicates primary resistance. Therefore in the future, the applicability of these drugs for individual patients will likely be based on immunohistochemical staining results of tumour samples. From experience with targeted drugs in more prevalent types of cancer, it is known that target expression can change during disease progression and that (re)sampling of multiple lesions has major impact on the choice for a therapeutic regimen (9, 10).

We aimed to determine whether heterogeneous tumour cell biology is also an issue of concern in ES. Because the best indicator for response to IGF-targeted therapy remains to be determined and the development of other targeted drugs for ES is foreseen in the near future, we examined heterogeneity by DNA content analysis with image cytometry, a well-founded and easy to use technology. Because the prognostic significance of DNA content in ES has been reported (11, 12) and this supports the validity of DNA content analysis for the detection of biologically different tumour clones, this was also assessed in our cohort.

Materials and Methods

A database was constructed containing data of all patients treated for ES between 1985 and 2005 at our Centre. Formalin-fixed paraffin-embedded archival tumour specimens were retrieved and the diagnosis was reviewed by a pathologist (PS). Only specimens containing over 50% of viable tumour cells and showing adequate

Correspondence to: P.J. Slootweg, MD, Ph.D., 824 Department of Pathology, Radboud University Nijmegen Medical Centre, P.O. Box 9101, 6500 HB Nijmegen, the Netherlands. Tel: + 31 243614323, Fax: + 31 243668750, e-mail: p.slootweg@pathol.umcn.nl

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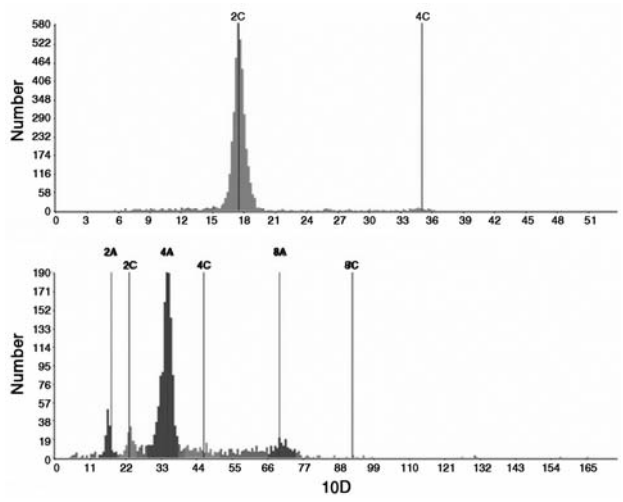

Figure 1. *DNA histograms. Top panel: diploid DNA content, bottom panel: aneuploid sample.*

Table I. *Change of ploidy state observed over time in cases 9, 41 and 51.*

Case	Therapy-naive biopsy sample	Resection after neoadjuvant therapy	Metastasis	Recurrence
9	Diploid	Aneuploid	Diploid	
41	Diploid		2 Aneuploid, 3 Diploid, 1 Tetraploid, 1 discarded	
51	Diploid	Aneuploid	2 Diploid	Diploid

Table II. *Multiple ploidy states coexisted in simultaneously resected metastases in case 41.*

Type	Site	DNA content	CGH array
Biopsy	Sacrum	Tetraploid	
Metastasectomy	Lung	Diploid	+4, +18, +20
	Lung	Aneuploid (discarded; CV>6%)	
	Lung	Tetraploid	+4, +18, +20
	Lung	Tetraploid	
	Lymphnode	Diploid	No aberrations
Metastasectomy	Lung	Aneuploid	

preservation of histology after processing (assessed on haematoxylin/eosin-stained sections) were selected for analysis. In most cases, multiple specimens (*e.g.* biopsy for diagnosis, resection of the primary tumour and resection of metastasis) were available; these were all assessed accordingly.

Ploidy analysis. For DNA image cytometry (IC), at least two 50 μm-thick sections were obtained of each sample followed by a 4 μm haematoxylin/eosin-stained section to check for representativeness. The method described by Hedley (13) with additional tissue dissociation by passing it through a syringe needle was used for cell nuclei extraction. The obtained suspensions were filtered with a Partec 50 μm CellTrics filter (Partec, Münster, Germany) and prepared as a monolayer by cytospin. Nuclei were stained with Feulgen's stain and periodic acid Schiff (PAS). The samples were evaluated using a cytometric image analysis system (QPATH; Leica Imaging Systems Ltd Cambridge, UK) that consists of an automated microscope with a digital camera connected to a computer that runs DNA analysis software. Of each specimen, 5000 nuclei were scanned and a minimum of 2000 images of tumour cell nuclei were manually selected, avoiding debris, necrotic or cut cells, and cell clusters. Lymphocytes were used as the standard internal controls. Histogram quality was evaluated by integrated optical density (IOD) values and its coefficient of variation (CV); $15 \leq IOD \leq 25$ and $CV \leq 5\%$ were considered acceptable (Figure 1). Criteria for ploidy classification were taken from the guidelines of the European Society for Analytical Cellular Pathology (ESACP) (14). Three of the measured specimens had been decalcified; this did not influence evaluability (15).

Bacterial artificial chromosome-comparative genomic hybridization (BAC-CGH) array. In one case with a particularly large number of specimens obtained at a single timepoint, heterogeneity was confirmed by BAC-CGH array. Genomic DNA was isolated from 1-4 50 μm-thick paraffin-embedded tissue sections, depending on the size of the tumour. Sections were deparaffinated 3 times for 5 min in xylene, rehydrated in 100, 96 and 70% ethanol for 30 seconds each, stained with haematoxylin for 40 seconds, rinsed with water and incubated overnight in 1 molar NaSCN at 37≥C to remove crosslinks. Slides were rinsed twice for 10 minutes in phosphate-buffered saline at room temperature. Tumour tissue was scraped from the slide with a scalpel to obtain at least 70% tumour cells in 200 μl Qiagen ATL buffer (QIAamp DNA extraction kit, Qiagen; Alameda, CA, USA), transferred to eppendorf tubes and incubated with 27 μl proteinase-K (20 mg/ml stock) at 450 rpm (Eppendorf Thermomixer) at 56˚C. Three more aliquots of 27 μl proteinase-K were added at 4, 20 and 28 h. After a total proteinase-K incubation of ~44 h, DNA isolation proceeded as in the manufacturer's protocol (Qiagen, Cat 51306). Samples of isolated genomic DNA were analysed by 0.8% agarose gel electrophoresis to visualize DNA concentration and size distribution. A male and a female pool of array CGH reference DNA was isolated from paraffin-embedded tonsil/spleen tissue of four cytogenetically normal individuals. Array CGH was performed on microarrays containing 30,000 Fluor-escence *in situ* hybridization (FISH)-verified BAC clones at a resolution of ~100 kb. Array preparation, labeling, hybridization, and scanning procedures were performed as described elsewhere (16).

Statistical analysis. Descriptive statistics are used to present the data. Overall survival was calculated from histological diagnosis to event or March 1, 2010 as arbitrary date of follow-up termination using the method of Kaplan and Meier. Groups were compared with the log-rank test for univariate analysis; multivariate analysis was omitted because of the small sample size. A p-value <0.05 was considered statistically significant and all tests were two-sided.

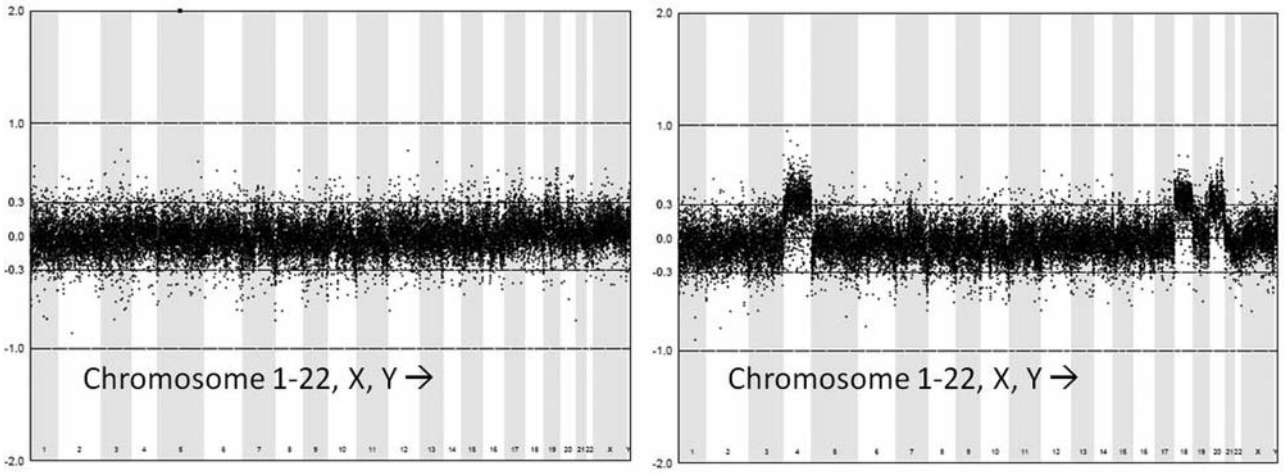

Figure 2. *BAC-CGH array in two synchronously resected tumour samples from case 41. Clones are arranged in the order from chromosome 1 to 22, X, and Y on the X-axis. On the Y-axis, the log2-transformed tumour over reference DNA fluorescence ratios are indicated. Left panel: no gains or losses are observed in a diploid lymph node metastasis. Right panel: a gain of chromosomes 4, 18 and 20 was observed in a tetraploid lung metastasis. A diploid lung metastasis had an identical chromosome pattern (not shown).*

Results

The Radboud University Nijmegen Medical Centre had 62 cases of Ewing's sarcoma between 1985 and 2005. From these cases, 42 samples from 21 patients met the aforementioned eligibility criteria for ploidy analysis. An additional 10 out of the 42 obtained DNA histograms did not meet our quality criteria and were discarded. Therefore 32 samples from 15 patients were included in this study.

At the date of analysis, 9 out of 15 (60%) patients had died (all of disease), 3 (20%) were alive with disease and 3 (20%) were disease free. The median follow-up of surviving patients was 103 months (mean 120.6, range 62-169, SD 46.3 months).

Of the evaluated tissue samples, 16 (50%) were diploid, 6 (19%) aneuploid and 10 (31%) tetraploid. Seven patients (44%) had a diploid biopsy sample and 5 (31%) an aneuploid one; 2 patients (13%) had a tetraploid biopsy sample. It was possible to assess change of ploidy in eight patients. In 2 cases, all tested specimens were diploid, 2 other cases retained tetraploid DNA content, 1 case was continuously aneuploid. In cases 9, 41 and 51, changes occurred as presented in Table I. There were two cases (5 and 41) with multiple metastases resected at a single timepoint. All samples in case 5 were diploid. However, in case 41, 2 out of 5 metastases were diploid, 2 tetraploid, and 1 aneuploid-hypodiploid histogram was discarded for quality reasons (Table II). The array CGH analyses performed on two samples from case 41 confirmed the existence of heterogeneous cell biology (Figure 2).

In patients with a diploid biopsy sample, the median overall survival was 84 months *versus* 19 months in patients presenting with a non-diploid tumor (p=0.047), while other known prognostic factors such as location (axial *versus* extremity), size of the primary tumour and presence of metastasis at diagnosis did not significantly influence prognosis on univariate analysis.

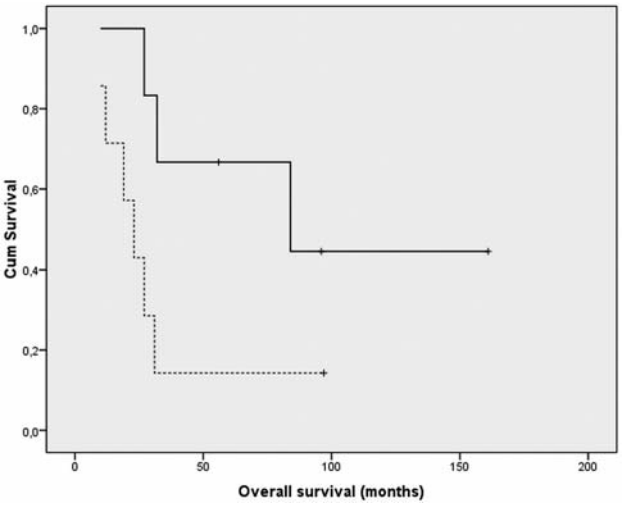

Figure 3. *Kaplan-Meier curve. In patients with a diploid biopsy sample (solid line), median overall survival was 84 months versus 19 months in patients presenting with a non-diploid tumour (dotted line, p=0.047).*

Discussion

Specific chromosomal translocations resulting in chimeric transcription factors that include motifs derived from unrelated genes may exhibit altered transcriptional activities. ES is characterized by such tumour-specific

translocations (*EWS-FLI1*: 95%, *EWS-ERG* 5%, others <1%) (17) which activate the *IGF1R* promoter (18-20), induce IGF1 expression (18-20) and alter IGFBP-3 (18, 20) and -5 (18) expression. ES is regarded as being strongly dependent on the IGF system (21) and the first clinical trials using IGF-targeted therapy show promising results (3, 4).

Secondary chromosomal aberrations in ES other than the pathognomic translocations frequently occur. Detailed analysis by CGH arrays (11, 22-26) show that gains are more frequently observed than losses, with gain of chromosomes 8, 12 and 1q as the most common findings. A more basic assessment of overall DNA content is possible by two techniques: IC and flow cytometry (FC). For both methods, a cell suspension of a tumour section is made. Whereas in FC the optical density of this suspension as a whole is measured, IC offers the possibility for selection; thereby debris, necrotic or cut cells, and cell clusters are removed. This makes IC more specific in the identification of tetraploid cell lines and more sensitive for smaller aneuploid populations (12). The legitimacy of this simplified approach to discriminate biologically different clones is supported by the work of Dierick *et al*. who showed that mortality in patients with a diploid ES was 42%. while it reached 100% in those with non-diploid ES (p<0.01) (12). Roberts and co-workers have also shown that a chromosome number above 50 (p=0.05) or a complex karyotype (p=0.04) are associated with worse overall survival (11).

We have successfully determined ploidy values by IC in 31 archival paraffin-embedded formalin-fixed ES samples from 15 patients. Allthough not the primary focus of our study, we were able to confirm that non-diploid DNA content is associated with worse overall survival, as suggested in literature (84 months *versus* only 19 months, p=0.047). This finding confirms that tumours with clinicaly different behaviour can be discriminated by ploidy measurement. While there is no internationally recognised risk classification system for ES, several factors, including axial or pelvic tumour site, large size and the presence of metastasis, are regarded as prognostically unfavourable. Unfortunately, it was impossible to perform a multivariate analysis of our small cohort to correct for these factors; none of these factors, however, influenced survival by univariate analysis (data not shown).

We found that in different tumour samples obtained over time from a single patient, DNA content can differ. Furthermore, in one out of two cases that allowed for the analysis of multiple metastases resected at a single timepoint, several co-existing ploidy states were observed. Aneuploidy is thought to emerge after tetraploidization of a diploid clone (27). This theory can explain the evolution of a diploid tumour to tetraploid or aneuploid lesions over time. However, in three cases, diploid lesions were found after previous detection of aneuploid lesions. These anomalies may be explained by chemosensitivity-based selection; a change in microenvironment can result in replacement of all tumour cells with the progeny of an innately drug-resistant variant cell (27). This is consistent with the fact that all ES with evidence of a change of DNA content over time have been subjected to chemotherapy and/or radiotherapy (28, 29). To the best of our knowledge, the coexistence of different ploidies in synchronously removed tumour manifestations has never been described in ES. Obviously, the small size of our study limits the implications of our findings. Furthermore, we used ploidy state to determine whether cell biology heterogeneity differs in ES rather than a direct predictor for response to IGF-targeted therapy. Since the best predictor for response to IGF-targeted therapies remains to be determined and we confirm that ploidy state successfully discriminates ES with different clinical behaviour, our findings strongly indicate that cell biology in ES may differ between different samples taken from a single patient. Screening for targets for novel therapies on a single tumour sample may therefore be insufficient and may lead to under- or overtreatment of patients.

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