ELSEVIER

European Journal of Cancer 40 (2004) 1945–1950

European Journal of Cancer

www.ejconline.com

Interpretation with hindsight

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Received 6 January 2004; received in revised form 12 January 2004; accepted 21 January 2004

Abstract

In his 1965 paper 'DNA content of tumours: cytophotometric measurements', Sandritter was for the first time able to relate tumour DNA content to the pathology and progression of a small number of tumours. In subsequent publications, these observations were extended to the progression of a much more comprehensive range of tumours. The interpretation of Sandritter's paper below follows the increasing sophistication of methodologies for tumour DNA content through the existing publications and evaluates the conclusions and hypotheses Sandritter proposed in the light of the contemporary account.

Keywords: Tumours; DNA content; Cytophotometry; Aneuploidy; Flow cytometry

1. Introduction

One of the earliest quantifications of the nuclear DNA content of tumour cells by cytophotometry was by Lapham in 1959 [1]. Thus, using what was still a relatively new technique in 1965, Sandritter was able to demonstrate, in his paper in the first edition of the *European Journal of Cancer*, that a range of human tumour cell nuclei exhibited an atypical distribution of DNA content. The technique of cytophotometry is based on Feulgen staining of nuclei either in tissue sections or after spreading of cells on to glass slides, followed by the quantitative measurement of photon emissions from the Feulgen dye across the cell nucleus after illumination with ultraviolet light. Perhaps surprisingly, essentially similar cytophotometric methods of estimating DNA content from Feulgen-stained nuclei are still very much in use (see below), although the estimates of staining density are based on digital-imaging cameras and software that allow much more rapid sampling of many more nuclei. By using initially tissue sections of small cell carcinoma of the lung and analysing the nuclei from the carcinoma cell chords, Sandritter was able to observe that in most cases the majority of *c* values were particular to each tumour and lay between either diploid/tetraploid or tetraploid/octaploid. These analyses also showed that the distribution of *c* values was similar in the peripheral zone as well as in the centre of the cell chord. He extended these observations to cell spreads of carcinoma *in situ* of the cervix. In contrast to the DNA content of simple atypical dysplastic epithelium, which had a proliferating diploid phenotype, an aneuploid DNA content was seen in the eight cases of carcinoma of the cervix studied. As with small cell carcinoma, what Sandritter called a stem-cell line, characteristic of individual tumours, was found in all layers of the carcinoma, and persisted through five different progressive stages of cervical carcinoma *in situ*. Similarly, in a single case of malignant lung adenomatosis, the 'stem-cell line' was constant, irrespective of the site of the malignancy, whether intra-alveolar or after metastasis to the lymph node. Finally, using a rat fibroblast model that had undergone malignant transformation *in vitro*, Sandritter observed that the aneuploid characteristics of the transformed cells *in vitro*, which remained stable after many months in culture, changed on transplantation into primary recipients and then underwent a subsequent change on secondary transplantation.

The data in this paper of 40 years ago are complex and difficult to interpret. If the consistency of the major 'stem line' within an individual tumour does indeed represent a stem-cell phenotype, then the implication is

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doi:10.1016/j.ejca.2004.01.043

that those cells of greater ploidies within the stem line represent differentiating tumour cells that probably are not proliferating. Conversely, the changes in the aneuploid phenotype of the rat fibroblasts from *in vitro* to *in vivo* and on subsequent transplantation presumably represent some form of tumour progression with associated genetic changes. In a later paper reflecting the rapidly increasing interest in the correlations between aneuploidy and nuclear DNA content in tumours, Bohm and Sandritter [2] published data from a very large study in which they were able to examine 165 smears from 105 different tumour cases from autopsy or surgical material. Their conclusions have in many ways set the scene for subsequent work in this area. A summary of them is that benign tumours were always diploid, in contrast to the vast majority (>95%) of malignant tumours, which were greater than diploid. Well-differentiated tumours, in particular those such as differentiated adenocarcinomas of the colon and endometrium, were in the hyperdiploid/triploid range (G1), with duplication peaks of 5*c* and 6*c* (G2). Conversely, anaplastic tumours, which histologically lack differentiated structures, do not display a homogeneous 'stem line' of DNA values, often exhibiting a broad peak in the hyperdiploid/tetraploid range. These kinds of tumour were often from autopsy samples, leading Bohm and Sandritter to the conclusion that the absence of a discernible 'stem line' was a predictor of endstage disease. Further, they concluded that in an individual tumour after metastasis the histological appearances and DNA contents of tumour cells were often congruent, irrespective of tumour site; however, in those cases where DNA contents did differ in different sites, the patterns of histology also shifted, usually to a more undifferentiated phenotype. A final conclusion was that precancerous conditions of the uterine cervix and the skin often contained cells with very high DNA contents (up to 32*c*), and that these could progress to 'carcinoma' *in situ*. Invasive growth of these carcinomas was associated with the selection of a stem line with a lower ploidy, which again persisted as long as the tumour was differentiated histologically but reverted to a broad, unimodal aneuploidy with the appearance of a dedifferentiated invasive carcinoma.

A very recent paper [3] applied essentially the same cytophotometric assay for Feulgen staining, but now with detailed standardised protocols that involve the analysis of over 200 nuclei in each preparation with internal '2*c*' reference cells, to assess the impact of oxygenation status on the DNA content of cells in cancers of the uterine cervix. These investigators' conclusions rebut those of earlier studies in showing no correlation between oxygenation status, thought from the earlier data to be a driver of aneuploidisation, and DNA content. Interestingly, the data did reveal a correlation between increased ploidies in precancerous lesions and patient age. From the perspective of the present article, these data are perhaps not as important as the demonstration that the issues that Sandritter's earliest observations raised in the 1960s are still reflected in much of the current debate about the existence of tumour stem cells, the association of changes in aneuploidy with tumour progression, and the relation between prognostic outcome and chromosomal phenotype. Sandritter's speculation in the earlier paper that there may also be some qualitative differences in 'nucleo-protein' content of normal as compared to neoplastic cells is also reflected in current attempts to refine tumour classifications and to predict more accurately treatment outcomes by microarray and proteome analyses of tumour populations. With hindsight and the availability of more sophisticated techniques for the measurement of DNA changes, the data in that paper are relatively crude. Because the numbers of nuclei measured were quite small for each tumour – mostly less than a hundred for each DNA measurement class – it is difficult to put any statistical certainty on the claim that the distribution patterns of nuclear DNA content within different components or stages of a single tumour are really similar, or simply reflect patterns of chromosomal instability in which large variations in DNA content of nuclei are incompatible with cell survival. Another limitation of this kind of DNA measurement is that no other information about the cell being assayed can be measured, so that correlation between even simple morphology with DNA content is not possible. Nevertheless, the questions that Sandritter and colleagues were trying to address are still extant and many tools such as flow cytometry, karyotypic analyses using chromosome painting and comparative genomic hybridisation are currently being used to unravel the relations between abnormalities in DNA content in the broadest sense and the malignant phenotype.

2. Flow cytometry

van Dilla *et al.* [4], in Los Alamos, developed a simple flow-cytometric method capable of the analysis of the DNA content of Chinese hamster ovary cells at rates of up to 50,000 cells/min, based on the fluorescence of Feulgen-stained cells in aqueous solution. This flow-cytometric technology was originally developed for the analysis of subatomic particles and in the late 1970s led to the development of flow cytometers specifically for the analysis of various histopathological characteristics of cells. Measurements of the DNA content of individual cell nuclei were then accomplished, using staining with intercalating dyes such as propidium iodide and ethidium bromide, and more recently dyes of the diamino-2-phenylindole (DAPI) and Hoechst series. More recently still, cyanine-type DNA reagents based on thi-

azole orange have been modified to increase their binding strengths [5]. Some of these, for example, are almost non-fluorescent in aqueous solution, but intercalation with DNA alters their chemical conformation to give relatively high quantum yields of fluorescence. With the concurrent development of ever more sophisticated fluorescent dyes and the increased multiparameter capabilities of flow cytometers, it is becoming possible to begin simultaneous large-scale analyses of tumour DNA content (even based on specific chromosomal or gene amplification) and the phenotype of the cell. One recent development describes a method for three-colour immunophenotyping and simultaneous measurement of DNA for comparison of normal peripheral blood and bone marrow with marrow from multiple myeloma (MM) patients, using a flow cytometer with one laser and an ultraviolet lamp [6]. In this case, DAPI was the DNA stain of choice, with fluorescein isothiocyanate, phycoerythrin and PC-5 (phycocyanine) as the fluorochrome conjugates for their diagnostic antibodies. All three of the fluorochromes were excited by a single argon laser at a wavelength of 488 nm. This is a significant advance, since before to these developments, aneuploidy detection by flow cytometry was largely informed by biparametric measurements simultaneously analysing DNA content and one antigen stained with an antibody [7,8]. One of the significant advantages of multiparameter analysis in haematopoietic malignancy is that a truly internal, G0/G1, normal diploid standard can be established, for example by measurement of the unaffected T-cell population and the malignant B-cell population simultaneously. In MM, many patients present with plasma cells that have either altered antigen expression compared to normal plasma cells or several different cell subpopulations. Aneuploidy within these myeloma subpopulations is often heterogeneous, some plasma cell subpopulations often having differing DNA contents. Thus, triple immunophenotyping and simultaneous DNA typing allows the fine analysis of these subpopulations and may allow the exploration of populations with differential sensitivity to, say, chemotherapy, possibly a more sensitive monitoring of minimal residual disease, and perhaps a definition of what may be a genuine 'stem line' in such malignancies.

Even without the benefit of such multiparameter analyses, DNA measurement by flow-cytometric methods is now commonly included as an element in the diagnosis, prognosis and follow-up of many 'solid' tumours as well as the more accessible haematological malignancies. The following articles exemplify the range of flow-cytometric studies that have been undertaken over the last five years to try and correlate DNA content with other clinical parameters of tumour progression. In lung cancer [9], flow-cytometric determination of DNA content appears to have prognostic value in that survival time after surgery was significantly shorter in patients with originally aneuploid tumours than those with diploid tumours, in spite of the fact that DNA content did not correlate with the frequency of metastasis, size or location. The investigators concluded that estimates of DNA content had some prognostic significance in terms of post-operative survival. In oral cancer [10], some workers have shown a good correlation between histological scoring of malignancy and DNA content, although in this disease, in contrast to some of Sandritter's findings, their was no statistical association between ploidy status and tumour differentiation. In a small study of glottic carcinoma [11], an attempt was made to correlate DNA measurements by flow cytometry with the macroscopic evaluation of the tumour by fibreoptic endoscopic examination. In this case, while there was a tendency for invasiveness to be associated with aneuploidy, the data were not robust enough to conclude that DNA content could replace macroscopic evaluation of the lesion, but taken together both observations may be prognostic for the radiation sensitivity of the tumour. Similarly, amongst human brain tumours [12], whilst the majority of benign tumours were diploid, malignant tumours with a higher incidence of aneuploidy and a higher DNA index were more likely to have a higher incidence of recurrence and a lower survival rate.

In ductal breast cancer [13], increased aneuploidy as measured flow cytometrically, and the increased expression of two transmembrane oncoproteins, EGFr and HER2/neu tyrosine kinase, were related to a more aggressive behaviour of the tumour. Aneuploid tumours might then have a higher number of chromosomal aberrations, leading to loss of cell-cycle control. This conclusion is reflected in a study of colorectal cancer in which a correlation was established between loss of p53 and aneuploidy [14], the inference being that the functional loss of p53 will predispose tumours to the development of aneuploidy and, by inference again, loss of cell-cycle control. These examples of the many publications are by no means definitive and there are many contradictory observations. Caution about the utility of the prognostic value of flow-cytometric determinations of tumour DNA content was expressed early in the development of these techniques [15]. Although a 'DNA-abnormal stem line', even in precancerous conditions, is indicative of neoplasia and should lead to a multiparameter assessment of tumour heterogeneity, DNA content itself may not be correlated with overt malignancy or an aggressive clinical course. Therefore, the prognostic impact of the measurement of aneuploidy has to be viewed in the context of other clinical features. Multiparameter flow cytometry measuring four or even five parameters simultaneously will undoubtedly help, but cannot in itself deal with the magnitude of diversity both within

and between tumours, and the role of DNA content as a factor in this diversity.

Interestingly, flow cytometry has been of use in the context of *hypo* diploidy and the role of apoptosis in the pathogenesis of human breast cancer [16]. Apoptotic cells appear in flow-cytometric analyses of DNA as a hypodiploid sub-G0/G1 peak as a consequence of partial DNA *loss*. Apoptotic cells can also be identified by flow cytometry through the terminal deoxynucleotidyl transferase nick-end labelling (TUNEL) technique, and the combination of cell-cycle and TUNEL analyses can relate the onset of apoptosis to the proliferation status of the tumour. In that study, apoptosis was found in aneuploid ductal invasive carcinomas and the presence of TUNEL-positive cells was correlated with the G2/S phase of the aneuploid cell-cycle. These observations, the investigators believed, may be indicative of p53 activity in these tumours and might suggest a need for modulation of therapeutic strategies in this class of tumours.

3. Karyotypic analyses: the chromosomal basis for aneuploidy

In the 1950s, it was realised that most tumour cell lines carried chromosomal abnormalities, but, because these abnormalities did not apparently correlate with tumour type, they were regarded as more a result of tumour transformation and subsequent chromosomal abnormality than in any sense causative. Although Giemsa and Wright staining techniques allowed the accurate grouping of chromosomes on the basis of size and shape, it was not until the 1970s and the development of the Giemsa-banding (G-banding) modification of Giemsa staining that translocation of chromosomes could be accurately identified and mapped (reviewed in [17]). Thus, the association of chronic myeloid leukaemia (CML) with the Philadelphia chromosome (a translocation between the end of the long arm of chromosome 9 and the long arm of chromosome 22 resulting in a much smaller than normal chromosome 22 – the Ph chromosome) was not finally established until 1972 [18,19]. Over the next two decades, work on Burkitt's lymphoma [20–22] and further work on the Ph chromosome [23,24] demonstrated that the translocations involved gave rise to fused mRNA transcripts of the two genes involved in the translocations, and that these particular translocations were associated with particular types of tumour and variations in the translocations associated with particular subtypes of leukaemia and lymphoma. More recent techniques, such as fluorescent *in situ* hybridisation (FISH), specific chromosome painting and spectral karyotyping (SKY), have made the identification of chromosomal aberrations involving specific genes or specific chromosomal segments much more accessible. Thus, it is now established that chromosomal abnormalities, especially translocations and duplications, are fundamental in many tumours, not only the leukaemias and lymphomas, to the progression of disease (reviewed in [25]). The cloning of two breakpoint genes in particular, AML-1 (also known as RUNX-1 or core-binding factor A2) and MLL-1 (ALL-1 or HRX), has now clearly demonstrated that the identification of genes involved at the breakpoints of chromosomal translocations may be a principal tool for the identification of genes critical to the progress of malignant transformation [25]. The former, AML-1, is a DNA-binding protein, which, after heterodimerisation to core-binding factor B, forms a complex that has high affinity for DNA. Activation of AML-1 is probably a 'master switch' for haematopoietic differentiation, and chromosomal abnormalities involving core-binding factors A and B account for about 30% of all leukaemias [26–28]. Similarly, MLL-1 has an important role in haematopoietic differentiation. It is homologous to the trithorax cluster of genes in *Drosophila* [29–31] and controls the activation of a large number of genes (>50) during mammalian differentiation; it may have a crucial role in the regulation of homeobox genes, including those involved in haematopoietic differentiation [32,33]. Like AML-1, different translocations involving the MLL-1 locus on chromosome 11 have been associated with acute myeloid leukaemia. To date more than 50 translocation partners of MLL-1 have been described and more than 30 of these partner genes have now been cloned. MLL-1 translocations account for between 10% and 20% of all acute leukaemias, with one translocation, the MLL-AF9 fusion, accounting for about a third of all MLL-1-associated acute leukaemias [25,34,35]. What is the relation between these translocations and aneuploidy in leukaemias? A chromosomal translocation *per se* will not fundamentally alter the DNA content of the cell involved. In those cases involving gene duplications or trisomies for specific chromosomes, the DNA contents may be altered in a marginal way, but the acute leukaemias, including those involving the genes described above, can also have many additional rearrangements and chromosomal aberrations. Thirty or more aberrations are not uncommon [17].

Again, the precise relation between the characteristic translocation, additional translocations and associated aneuploidy and leukaemic progression is unknown. Transgenic animal models of leukaemias with, for example, human MLL1-AF9 fusion genes translocated into the MLL-1 gene, and animals transgenic for AML1-ETO sequences, have shown that, in the former, acute myeloid leukaemias develop spontaneously, albeit with a relatively long latency [36,37], but with AML1-ETO a secondary mutational event is necessary [38]. These findings may be reflected clinically, in that in the case of 11q23/MLL-associated acute leukaemia only 22

out of 54 cases had additional chromosomal abnormalities and these had no apparent bearing on prognosis [39], whereas in cases of AML1-ETO-associated acute leukaemia, cells carrying the mutation are still detectable in the peripheral blood during complete remission [40]. Landritter's original questions about the correlations between stem-line aneuploidy and prognosis still require to be evaluated, even in the context of haematological disorders from which so much information about the causative effects of chromosomal aberrations has already been gleaned.

The association between specific chromosomal translocation and tumorigenesis is not confined to haematological disorders, where it has been relatively easy to analyse karyotypes longitudinally through presentation, treatment, remission and relapse, but is being increasingly confirmed in cases of solid mesenchymal and epithelial tumours. Cases in which there are chromosomal abnormalities in tumours that recur in at least several cases are now being recorded in a catalogue maintained by the Mitelman group in Sweden as well as by the National Cancer Institute [41].

In a recent review, Rajagopalan *et al.* [42] have explored the experimental and theoretical evidence for the role of the generation of chromosomal instability in the progression of colorectal cancer. Colorectal cancer is thought, in the majority of cases, to be initiated by inactivation of the APC tumour-suppressor pathway, and, in over 90% of cases, genes in the pathway are mutated [43,44]. This primary event leads to the dysplastic growth of mutant cells in the stem-cell areas of the gut crypts and the accumulation of polyps. Some 10–20% of the large polyps progress to malignancy over the next 20–40 years, through a variety of genetic changes including the generation of chromosomal instability [45]. These investigators argue that mutations leading to the generation of chromosomal instability are very early events in the tumour's progression and that it is through such a mechanism that individual cells accrue the genetic changes necessary to give them the selective advantage to outgrow other cells in the tumour. They demonstrate, in support of this hypothesis, that aneuploidy, as a correlate of chromosomal instability, is an early event in the formation of adenomas in the gut, and postulate that instability in itself may be responsible for the accrual of sufficient mutations to achieve tumorigenic status. It follows from their hypothesis that chromosomal instability should not cease as soon as sufficient genetic change has accrued. This may explain the heterogeneity of aneuploidy within tumours, and may also explain Sandritter's observations of the maintenance of a 'stem line' within the tumour mass.

The accumulation of data on the nature of genetic change leading to cancer has been vast in the 40 years since Sandritter's *European Journal of Cancer* paper and it is clear that this fundamental relation between aneuploidy, tumour progression and prognosis is still not fully understood. The key to the understanding of these phenomena may lie in our increasing ability to sort small numbers (if not single cells) of homogeneous cells from the tumour mass based on their DNA content and phenotype, and to perform gene expression, proteomic and even 'metabolomic' analyses on these populations to unravel the important elements of the uncontrolled growth of these cells.

Meanwhile the issues raised by Sandritter still provoke much activity and heated debate.

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