Introduction

Electrophoresis of enzyme-digested DNA has been undertaken largely as one-dimensional separations. Several groups have sought to enhance resolution by separating DNA fragments in two dimensions. Fischer and Lerman [1] relied on two independent separation modes, size in the first dimension and mobility in a denaturing gradient in the second dimension, to separate DNA restriction fragments. This two-dimensional (2-D) separation system has been further developed for human genomic DNA typing, by adding a hybridization step using minisatellite core probes [2]. A probe-free method was developed by Yi et al. [3] to detect DNA rearrangements in bacteria, based on the electrophoretic separation of enzyme-digested DNA in the first dimension, followed by in situ digestion with an additional restriction enzyme before the second-dimension separation. Likewise, Hatada et al. [4] demonstrated that by radioactively labelling genomic DNA at cleavage sites specific for a restriction enzyme, followed by first-dimension separation and in situ digestion in the gel before second-dimension separation, a high degree of resolution could be achieved.

We have implemented a computerized approach initially developed by our group for protein 2-D analysis [5–7] for the analysis of 2-D separations of enzyme-digested genomic DNA [8,9]. By utilizing different combinations of restriction enzymes and/or different electrophoretic conditions, the number of independent fragments in a human genomic DNA sample that can be analysed in multiple 2-D patterns can reach several thousand. The approach relies on radioisotope labelling of genomic fragments at cleavage sites specific for a rare cutting restriction enzyme. The labelled genomic digests are separated in a first dimension, followed by in situ digestion before second-dimension separation. The reliance on the rare cleaving restriction enzyme NotI to digest genomic DNA before labelling, allows visualization of DNA fragments that occur preferentially in CpG islands of the genome. Because of the localization of CpG islands in proximity to transcribed sequences, the 2-D patterns obtained with this enzyme are highly targeted to a functional component of the genome [10,11]. Thus there is a strong likelihood that NotI fragments detected in 2-D gels represent sequences in genes.

An important application of this approach is the study of genomic alterations in cancer. Tumour cells display a wide range of genomic alterations including chromosomal rearrangements, deletions, amplifications and point mutations. In addition, epigenetic changes as may result from altered DNA methylation have been observed in tumours [12]. Altered DNA methylation at specific sites, in addition to its potential direct effect on gene expression, may affect mutational events at these sites including rearrangements and point mutations [13–15]. Here we review our approach for 2-D DNA analysis as applied to neuroblastoma and discuss our findings of genetic alterations in this tumour type.

Neuroblastoma is a childhood cancer that originates in cells from the neural crest. The clinical course of the disease varies widely [16].

Abbreviation used: 2-D, two-dimensional.
*To whom correspondence should be addressed.

References:


Received 24 July 1996

Identification of amplifications, deletions and methylation changes in cancer by means of two-dimensional analysis of genomic digests: application to neuroblastoma

K. Wimmer, D. Thoraval, R. Kuick, B. J. Lamb and S. M. Hanash*
Department of Pediatrics, University of Michigan, Ann Arbor, MI 48109-0510, U.S.A.
Several non-random genetic abnormalities have been identified in neuroblastoma cell lines and primary tumours. These include allelic loss of chromosome 1p [17-20] and N-myc oncogene amplification. Several lines of evidence suggest that the cytosine methylation status of certain loci or chromosomal regions may affect the development of neuroblastoma or contribute to its phenotype [21]. Identification of loci that undergo methylation changes in the genome of cancer cells, apart from the analysis of candidate genes, has been limited by the lack of suitable means to search for methylation alterations across the genome. Two-dimensional electrophoresis of genomic DNA allows the detection of amplifications, deletions and methylation changes by measuring fragment intensities. We have previously shown that loss of one of the two genomic copies encoding for a fragment could be detected without the need for reliance on heterozygosity [8,9]. Thus the 2-D electrophoresis of genomic digests could be useful for genomic amplification, deletion and methylation studies.

Materials and methods
Preparation of 2-D gels of genomic DNA
For the studies described, whole genomic DNA of tumour tissue and normal control tissue or cell lines, as well as single-chromosome preparations obtained by flow sorting, is digested with the enzyme NotI and a second 6bp cutting enzyme such as EcoRV or BglII [9,22]. The protruding ends produced by the methylation-sensitive CpG-specific enzyme NotI are isotopically labelled. About 1 μg of the resulting DNA fragments or the resulting fragments of 2 × 10^4 flow-sorted chromosomes are subjected to disc-agarose-gel electrophoresis to separate fragments in the 1-5 and 5-12 kb range. The separated DNA fragments are subsequently subjected to further in situ cleavage with a third frequently cutting restriction enzyme (HinfI or PstI) and electrophoresed in a second dimension in polyacrylamide gels. The resulting 0.3-2.0 kb fragments are visualized using phosphor storage technology. The digitized images are analysed using software we have previously developed [5-7].

Cloning of DNA fragments
DNA fragments may be cloned directly from preparative gels [23] as briefly presented here for NotI-PstI fragments. Both radiolabelled and non-labelled genomic digests are loaded on to first-dimension agarose gels. After the second dimension in polyacrylamide, the gels are exposed to X-ray film (RX; Fuji) for 24 h at −80°C. DNA fragments of interest are recovered by elution with high-salt elution buffer (50 mM Tris/HCl, pH 8.0, 1 M NaCl, 10 mM EDTA) and the DNA is ligated in a NotI-PstI-digested pBluescript vector in the presence of DNA ligase at 16°C for 40 h. The transformation is performed by electroporation using electroporation-competent cells.

Results
Identification of hypomethylated repetitive DNA sequences
To date we have analysed 19 neuroblastoma tumours and six neuroblastoma cell lines by means of 2-D DNA electrophoresis. We have observed a series of multicopy fragments that are absent from normal tissue. Three of such fragments, denoted Nbl-1, Nbl-2 and Nbl-3, have been described in previous studies [23-25]. Cloning and sequence analysis of fragment Nbl-1 revealed strong homology between this fragment and a subtelomeric sequence reported to occur in chimpanzees but was not detectable in humans [26]. Further analysis [23] of this fragment showed that it is part of a repetitive sequence that is subjected to methylation in normal tissue, and the occurrence of this fragment in tumour DNA is the result of partial demethylation at NotI sites in the repetitive DNA. Furthermore identification of this sequence, which is also found in subtelomeric regions of all acrocentric chromosomes in human DNA, indeed indicated greater similarity between human and chimpanzee subtelomeric sequences than previously recognized.

A second fragment (Nbl-2) (Figure 1) was found to occur in multiple copies in neuroblastoma tissue and was characterized by Thoraval et al. [25]. A third fragment (Nbl-3) (Figure 1) was also detected in multicopies in neuroblastoma by our group [25] and in other tumours by another group [24]. Both fragments represent parts of repetitive sequences in the genome. The appearance of both fragments in 2-D gels in neuroblastoma DNA was demonstrated to be due to demethylation of cytosine at the NotI sites.

Identification of a novel amplified fragment in neuroblastoma
A 400 bp NotI-HinfI fragment that occurs in two-copy intensity in 2-D gels of control DNA...
Figure 1

Digital images of whole genomic DNA of a neuroblastoma tumour (B) and of peripheral blood cells of the same patient (A)

DNA was digested with NotI, EcoRV and Hinfl and separated in the 1–5 kb range in the first dimension. Two fragments, Nbl-2 and Nbl-3, are marked with arrows in the tumour tissue. Both fragments are not visible in normal control cells. Other multicopy fragments visible in both patterns are largely attributed to ribosomal DNA.

Figure 2

2-D image of genomic DNA cut with the enzymes NotI, EcoRV and Hinfl, separated in the 5–12 kb range

(B) Pattern of a neuroblastoma tumour with an amplified N-myc gene. An amplified fragment of the N-myc gene and an as-yet unidentified multicopy fragment are marked with arrows. (A) Normal control tissue not showing the multicopy fragments.

showed increased intensity in three neuroblastoma cell lines and three tumours, all characterized by N-myc amplification. This fragment is not part of the N-myc gene. The latter yields a different-size fragment with the enzyme combination utilized (Figure 2). The 400 bp fragment was cloned and yielded a novel sequence. Southern-blot analysis performed with the cloned 400 bp NotI–Hinfl fragment clearly showed that this fragment is amplified in tumour
tissue and cell lines in concordance with the 2-D DNA findings (K. Wimmer, D. Thoraval, R. Kuick, B. J. Lamb and S. M. Hanash, unpublished work).

Quantitative analysis of chromosome 1 NotI fragments in neuroblastoma

Flow cytometry has provided the means to purify single chromosomes [27]. In a previous study, we have shown that the availability of single-chromosome preparations allows the 2-D separation of restriction fragments of specific chromosomes and the identification of corresponding fragments in whole genomic patterns (Figure 3A) [28]. A total of 346 fragments in whole genomic 2-D patterns were assigned with high confidence to chromosome 1 based on their co-migration with chromosome 1 fragments. The intensities of 183 fragments assigned to chromosome 1 in the range 1–5 kb were investigated in neuroblastoma 2-D patterns. Reduction in fragment intensity or complete absence of a fragment could be due to deletion or cytosine methylation at its NotI site. Analysis of fragments in five neuroblastoma cell lines and four tumours and corresponding control tissues was undertaken. Nine chromosome 1 fragments were found to exhibit reduced intensity in the majority of the cell lines and tumour samples, compared with normal two-copy fragments and compared with their intensity in the control samples. Interestingly, five of these nine fragments exhibited reduced intensity in all tumours that showed deletion in the short arm of chromosome 1 by independent analysis. This suggests that these NotI chromosome 1 fragments might be derived from genes that are deleted in neuroblastoma. One of the fragments is shown in Figure 3.

Discussion

We have summarized here our experience with the 2-D electrophoresis of genomic DNA for the detection of genomic alterations in a cancer type. This approach provides the means to analyse several thousand genomic NotI fragments from a single DNA preparation. The availability of single-chromosome 2-D DNA preparations facilitates the assignment of individual fragments in whole genomic pattern to the chromosome they are derived from. Quantitative analysis of fragment intensities allows the detection of

Figure 3

2-D pattern of an Epstein–Barr virus-transformed cell line digested with NotI, EcoRV and HinfI separated in the 1–5 kb range

Fragments that are attributed to chromosome 1 are marked with white crosses (A). The frame indicates the area shown in the close-up for a neuroblastoma tumour (C) and control tissue of the same patient (B). A chromosome 1 fragment that exhibited reduced intensity in all tumours with chromosome 1p deletions is marked with an arrow.
amplifications as well as the detection of deletions and methylation changes in single-copy genes. The success of detecting deletions requires sufficient quantitative reproducibility in fragment intensity to detect loss (or methylation) of one of two genomic copies of a fragment. Because the fragments are visualized by means of end-labelling at restriction-site overhangs, the intensity of a fragment reflects the amount of incorporated radioactivity at its cleaved end and therefore the number of genomic copies per cell of each fragment in the DNA preparation. For 2-D patterns prepared using methylation-sensitive enzymes such as NotI, methylation at the restriction site of either of the two fragment copies is also detectable.

In our analysis of neuroblastomas, nine fragments exhibited reduced intensity relative to normal control tissue. Some of these fragments probably represent sequences of genes located on the short arm of chromosome 1. Deletions of chromosome 1p are associated with an unfavourable outcome in neuroblastoma [21]. The common region of deletion [17] is suspected to harbour one or more tumour suppressor genes. Our analysis of neuroblastoma has also uncovered demethylation of highly repetitive DNA fragments. There is some evidence suggesting that demethylation of the repetitive units we have identified occurs as part of tumorigenesis. We demonstrated in experiments using methylation inhibitors that demethylation of the repetitive units we observed did not result from labile methylation [25]. In addition, our studies [23,25] taken together with results published by others [24] indicate that the demethylation we have observed is not a feature of the tissue origin.

Our analysis has also uncovered a fragment that is amplified in neuroblastoma. Its co-amplification with N-myc suggests that it is a part of the N-myc amplicon; however, we have shown that this fragment does not belong to the N-myc gene and therefore probably represents an as yet unidentified gene. Further experiments to characterize this gene and its relevance to tumour development are in progress. The findings presented here illustrate the utility of the 2-D approach for cancer-related investigations. A database of genomic fragments that exhibit changes in different tumour types is currently being developed by our group.

K.W. gratefully acknowledges support of the Austrian Fond zur Förderung der wissenschaftlichen Forschung (Erwin-Schroedinger-Stipendium Nr. J 01089-MOB).

21 Caron, H., van Sluis, P., de Kraker, J., Bakkerink, K.W. gratefully acknowledges support of the Austrian
Recent advances in capillary electrophoresis of DNA fragments and PCR products
P. G. Righetti* and C. Gelfi†

*Faculty of Sciences, University of Verona, Cà Vignal, 37134 Verona, Italy and †TBA, CNR, Via Ampère 56, Milano, Italy

Introduction
We do not intend, in this review, to cover both the capillary zone electrophoresis (CZE) technique and all aspects of DNA separation by this methodology. There already exist ample reviews on this topic, to which readers are referred for information on the origins of CZE and applications to the vast field of DNA separations [1-3]. For those interested in the theoretical aspects and principles of size-based separation in polymer solutions, an in depth review also exists [4].

A review of the use of physical networks in CZE is also available [5]; in addition, the use of fluorescent reporters in CZE has been adequately covered [6]. There is a plethora of books dealing with CZE [7-19], and it is to be expected that, in 1996, a small avalanche will follow.

Our review will deal mostly with what we have developed in the field of CZE, in DNA separations ranging from oligonucleotides to PCR-amplified fragments for detection of genetic diseases. The review will end with the quantitative use of CZE for precise gene dosage and with the development of non-isocratic CZE for detection of DNA point mutations. Thus the DNA we will deal with is confined to short fragments, ranging from a dozen to a few hundred base pairs. We would like, in addition, to emphasize that today the vast majority of users have adopted liquid sieving polymers instead of true gel matrices, thus bringing to life an early dream of DeGennes [20]. In particular, we use N-substituted polyacrylamides [21-23].

Separation of antisense oligonucleotides
The production of high-purity oligodeoxyribonucleotides is becoming increasingly important in a wide variety of applications, ranging from their use as hybridization probes in diagnostic and gene cloning experiments, primers for DNA sequencing and for PCR, and reagents for DNA fingerprinting, to their utilization as ‘antisense’ therapeutics. Although improved chemical synthesis and automated instrumentation have greatly simplified the assembly of these molecules, the actual amount of the expected full-length product varies depending on coupling efficiencies and the length of the desired DNA product. The purity required of the DNA product varies according to the intended end use. When the oligodeoxyribonucleotide is used as a hybridization probe or primer, analysis and purification methods are seldom employed since low levels of ‘failure sequences’ are usually inconsequential to the end result. However, in several applications, such as site-directed mutagenesis, X-ray crystallographic and antisense investigations, use of oligonucleotide purification and ana-