

ORIGINAL ARTICLE

Comparative genomic hybridisation as a supportive tool in diagnostic pathology

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Aims: Patients with multiple tumour localisations pose a particular problem to the pathologist when the traditional combination of clinical data, morphology, and immunohistochemistry does not provide conclusive evidence to differentiate between metastasis or second primary, or does not identify the primary location in cases of metastases and two primary tumours. Because this is crucial to decide on further treatment, molecular techniques are increasingly being used as ancillary tools.

Methods: The value of comparative genomic hybridisation (CGH) to differentiate between metastasis and second primary, or to identify the primary location in cases of metastases and two primary tumours was studied in seven patients. CGH is a cytogenetic technique that allows the analysis of genome wide amplifications, gains, and losses (deletions) in a tumour within a single experiment. The patterns of these chromosomal aberrations at the different tumour localisations were compared.

Results: In all seven cases, CGH patterns of gains and losses supported the differentiation between metastasis and second primary, or the identification of the primary location in cases of metastases and two primary tumours.

Conclusion: The results illustrate the diagnostic value of CGH in patients with multiple tumours.

In day to day routine work, pathologists are faced with cases that take more than a standard haematoxylin and eosin stain to solve the diagnostic problem and answer the clinician's questions. Patients with multiple tumour localisations belong to a special category in this respect. Of course, in a large number of cases, in patients with a known and well characterised primary tumour, the second tumour can be confirmed—at least for all practical purposes—to be a metastasis of this primary. In fact, this decision in general is not only based on the histological comparison between the two tumour samples, but also on the clinical plausibility. For example, adenocarcinoma in the liver is regarded as a metastasis of the patient's colorectal cancer, the squamous cell cancer in a jugular lymph node as a metastasis of the patient's laryngeal cancer, and adenocarcinoma in an axillary lymph node as a metastasis of the patient's breast cancer. In other cases, the situation is less obvious, and the question of whether the patient has a metastasis of his primary cancer, or whether a second primary cancer has occurred, is difficult to answer, even though this may be very important for clinical decision making. In these cases, the question is: should the patient be regarded as suffering from (high stage) metastatic disease, or has a low stage second primary occurred, and should the patient be treated accordingly with an intention to cure? The pathologist plays a key role in this process of decision making and is expected to produce the ultimate answer. However, it is obvious that this is not always an easy task, and frequently additional techniques are required. Immunohistochemistry is often the initial method used, but is frequently not enough, either because certain immunophenotypes are common to several types of cancers, or because tumours are negative for almost all markers. In addition, different samples from the same tumour may show extreme phenotypical and immunohistochemical heterogeneity. Therefore, it seems logical to focus not only on the phenotype, but also to look at the genotype. In our study, we present several cases in which comparative genomic hybridisation (CGH) was applied in an attempt to reach a diagnosis.

CGH is a technique that allows the detection of chromosomal copy number changes without the need for cell

culturing.^{1–3} It gives a global overview of chromosomal gains and losses throughout the whole genome of a tumour. Thus, CGH is a relatively fast screening technique that can point at specific chromosomal regions that have been altered in the genome. Because no cell culturing is required for CGH, this technique has enabled tremendous progress in the analysis of chromosomal changes in solid tumours. Applications of CGH in cancer research include screening of tumours for genetic aberrations^{4–16} (see also <http://www.helsinki.fi/~lgl/www/CMG.html>), searching for genes involved in the carcinogenesis of particular subsets of cancers,¹⁷ analysing tumours in experimental models to provide more insight into tumour progression,^{17,18} diagnostic classification,¹⁷ and prognosis assessment.^{17–20} In addition to these oncological applications, CGH analysis has also been used to study chromosomal aberrations in fetal and neonatal genomes.^{21,22}

“Comparative genomic hybridisation gives a global overview of chromosomal gains and losses throughout the whole genome of a tumour”

CGH may prove to be useful for the differentiation between a metastasis or second primary, and the identification of a primary location in cases of metastases and two (or more) primary tumours. To investigate this possibility, we decided to analyse several cases with CGH and to look for the concordance of the chromosomal aberrations between multiple tumour samples within one patient.

METHODS

DNA was isolated from paraffin wax embedded, formaldehyde fixed tissue according to the protocol described previously.³ CGH was performed as described previously,³ and the results were analysed with an Applied Imaging workstation (Applied

Abbreviations: CEA, carcinoembryonic antigen; CGH, comparative genomic hybridisation; CK, cytokeratin

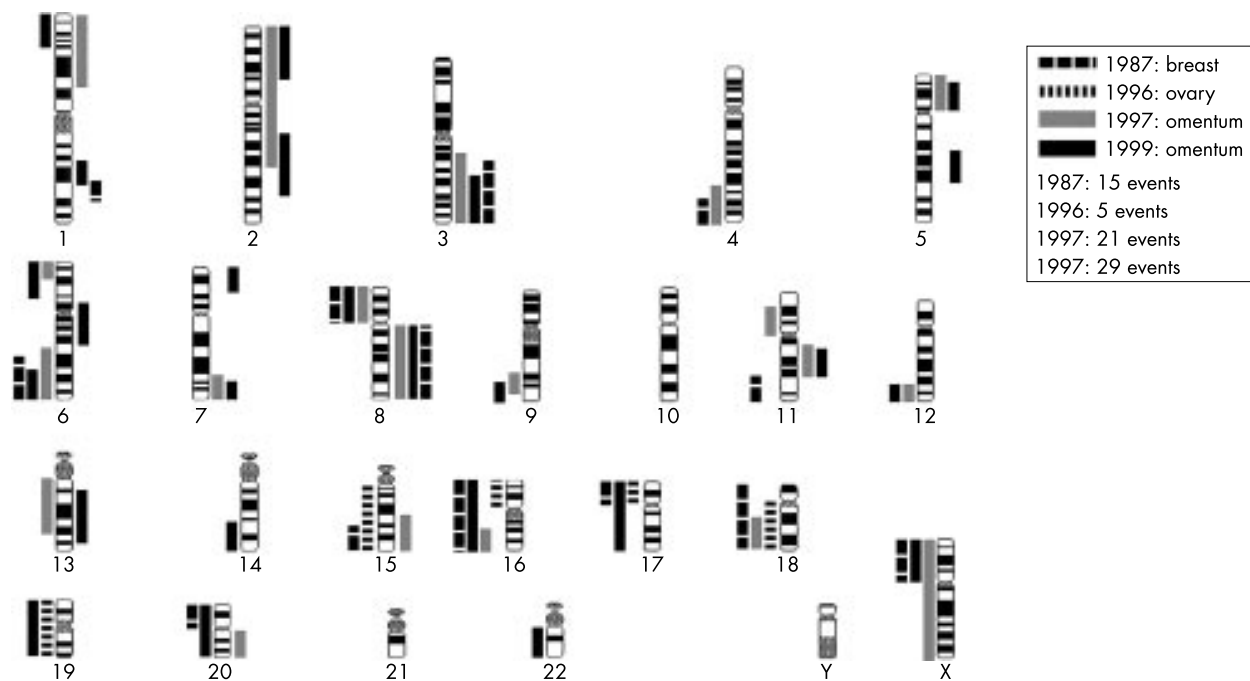


Figure 3 Overview of comparative genomic hybridisation events in the four lesions described in patient 2. The bars on the right of each ideogram represent gains, and the bars on the left losses.

results showed that the tumour in the gastric cardia and the tumour in the distal oesophagus were genetically unrelated and confirmed that the oesophageal tumour was a second primary, so that there were no indications of metastasis of the primary tumour.

Patient 2

A 51 year old woman presented with a medullary type of breast cancer that was locally radically resected. There were no lymph node metastases. Nine years later she presented with an 11 cm poorly differentiated adenocarcinoma of the left ovary and a 1.5 cm tumour with similar histology in the right ovary; no abdominal metastases were present. Another year later, she had a metastatic tumour removed from the omentum, which proved to be a poorly differentiated adenocarcinoma. Two years after that she underwent partial duodenectomy because of an obstructing tumour mass, which on microscopic examination also proved to be a poorly differentiated adenocarcinoma. Clinically, the ovarian tumours were thought to represent a second primary, although

histologically discrimination between the tumours (of the breast, ovary, duodenum, and omentum) was not completely straightforward (fig 2). BRST2 immunohistochemistry was positive in the breast tumour and the metastases in the omentum and duodenum. The two metastases had 13 CGH events in common (fig 3), and therefore were thought to have developed from the same primary tumour. The breast carcinoma from 1987 shared six events with the metastases, whereas none of these six occurred in the ovarian tumour from 1996 (fig 4). It was concluded that the two metastases were derived from the breast carcinoma and that the ovarian cancer was a second primary.

Patient 3

A 76 year old woman underwent a lumpectomy for a 3 cm large ductal adenocarcinoma in the right breast. The resection margins were free but the sentinel node showed a metastasis. Subsequent lymph node dissections of the right axilla yielded nine lymph nodes free of tumour. Three years later the patient presented with an undifferentiated carcinoma in the bladder. On immunohistochemical evaluation, both tumours were positive for cytokeratin, CAM5.2, AE1/3, and the progesterone receptor. In addition, the breast cancer was positive for the oestrogen receptor (bladder negative) and the bladder was positive for cytokeratin 7 (CK7) and CK20 (breast negative). Both tumours were negative for BRST2. CGH was performed on both the breast and the bladder tumour. The breast tumour showed many aberrations ($n = 25$), in contrast to the bladder carcinoma, in which only nine chromosomal changes were found. In addition, both tumours showed specific changes—for example, the breast tumour showed amplification at 1q31–32, and in the bladder carcinoma amplifications were present at both telomeres of chromosome 3 (a very rare amplification). This virtually excluded the possibility that both tumours shared a common origin.

Patient 4

At necropsy of a 91 year old woman, multiple tumour localisations were found in the supraclavicular lymph nodes, pancreas, and right breast. The patient had a history of an adenocarcinoma of the left breast for which she underwent a

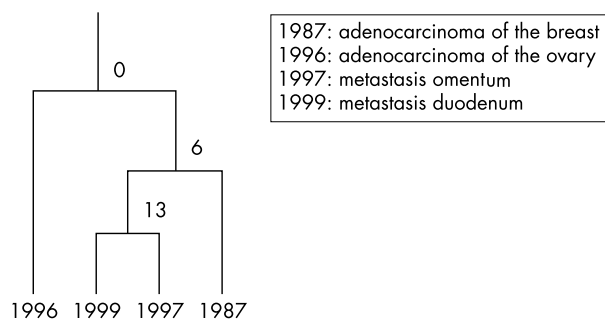


Figure 4 Summary of the results from patient 2. The two metastases have 13 comparative genomic hybridisation events in common and are therefore considered to have developed from the same primary tumour. The breast carcinoma from 1987 shared six events with the metastases, whereas none of these six occurred in the ovarian tumour from 1996. It was concluded that the two metastases were derived from the breast carcinoma and that the ovarian cancer was a second primary.

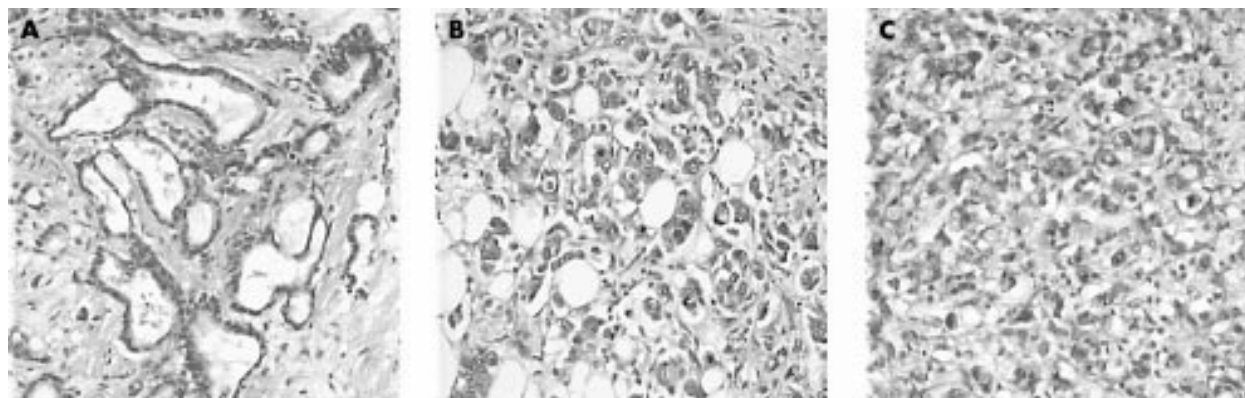


Figure 5 Histology of patient 4. (A) The breast lesion showed adenocarcinoma with predominant tubule formation and moderate cytonuclear atypia, whereas (B) the pancreatic tumour and (C) lymph node metastases both showed poorly differentiated adenocarcinoma, with solid small epithelial nests and moderate to strong nuclear atypia.

mastectomy 40 years before she died. The tumour in the pancreas was histologically different from the other tumours (fig 5), but immunohistochemistry was inconclusive. All tumour samples were positive for CAM5.2, CK7, AE1/3, and carcinoembryonic antigen (CEA) and negative for BRST2.

CGH analyses of primary breast cancers and metachronous metastases after several decades have been described.²³ In this patient, we performed CGH on the breast tumour, the pancreatic tumour, and one lymph node metastasis. Unfortunately, there was no material available from the breast tumour removed 40 years before death. The breast tumour showed 15 chromosomal aberrations, the pancreatic tumour showed nine chromosomal aberrations, and the lymph node metastasis 21. The breast and the pancreatic tumours had seven (of 15 and nine, respectively) events in common. The breast cancer and the lymph node metastasis shared nine (of 15 and 21, respectively) events, and the pancreatic cancer and the lymph node metastasis shared eight (of nine and 21, respectively) events. These CGH results indicated that all the lesions had developed from the same tumour. Based on the idea that the tumour with the least changes is the primary, this would be the pancreatic tumour, although theoretically it is possible that all the tumours found at necropsy had originated from the breast cancer diagnosed 40 years before death. However, clinically, such a long time interval before relapse is less likely.

Patient 5

A 59 year old woman presented with a tumour in the right ovary with clear cell histology. Seven years before she had undergone a nephrectomy because of a renal carcinoma of the left side. Both the combination of a primary renal cell carcinoma and an ovarian clear cell carcinoma, or the alternative, a renal cell carcinoma with a metastasis in the ovary, are rare. Furthermore, the pattern of tumour spread (along the para-aortal lymph nodes with no intra-abdominal metastases) is remarkable for an ovarian adenocarcinoma. Some differences were seen on histology and immunohistochemistry (the renal cell carcinoma was negative for CK7, CEA, CA125, and vimentin, whereas the ovarian tumour was positive for all these markers). CGH showed 11 chromosomal aberrations in the renal cell carcinoma and 25 in the ovarian tumour. Out of the total number of 30 different chromosomal aberrations, the tumours had only five in common. In this case, the combination of genotyping by CGH and immunophenotyping yielded sufficient information to classify the tumours as two independent lesions, and it was therefore concluded that the tumour in the ovary was a second primary.

Patient 6

A 72 year old man who underwent resection of the inferior lobe of the right lung because of a 5.5 cm tumour was found

to have an additional 1.1 cm tumour in the same lobe that was quite different histologically. The largest tumour had an epithelial undifferentiated large cell phenotype, whereas the smaller tumour showed a mesenchymal aspect, with polymorphic atypical spindle cells and many mitoses.

The total numbers of chromosomal aberrations detected by CGH were 17 and 19, in the larger and smaller tumours, respectively, of which they had 10 in common. Both lesions showed several chromosomal aberrations that are rather typical for squamous cell carcinomas,¹² such as gain of 3q and 11q13, and loss of 13q. Because similar levels of agreement have been seen within tumours, the CGH results were consistent with the two tumours being genetically related.²⁴

Patient 7

A 58 year old woman presented with synchronous endometrioid carcinomas of the ovary and endometrium. Both tumours were diploid as analysed with flow cytometry. CGH was performed on both lesions and revealed only three events in the uterus (8p-, 16q-, and Xp+) and two in the ovarian carcinoma (12q-, 22q-). Because none of these chromosomal aberrations was shared, it was concluded that these two endometrioid tumours were not related, and thus they were considered to be two primary tumours.

DISCUSSION

The traditional role of the pathologist has been the evaluation of human tumour samples in a search for clues to their histogenesis and anticipated clinical behaviour. Over the years, this quest has benefited from the application of specialised techniques, which have become part of the pathologist's arsenal. In addition to light microscopic evaluation of haematoxylin eosin stained tissue sections, immunohistochemistry has proved to be a particularly valuable adjunct in cancer diagnosis. More recently, molecular pathological techniques have been added to this repertoire of useful techniques.

Irrespective of the tools applied, every diagnosis in clinical pathology, as one of the laboratory disciplines, should be considered as the result of a diagnostic test. For such a test to make an optimal contribution to clinical decision making, ideally it should be validated and implemented in a standardised way.²⁵ This includes defining the algorithm to calculate the classifier, deciding on the optimal cut off point depending on the clinical relevance, and ultimately extensive testing of the performance of the test in terms of positive and negative predictive value. This process is very demanding in terms of labour and availability of clinically and pathologically well documented series of specimens, and consequently it takes many years. When the respective differential diagnoses (which actually means the different diagnostic tests) in every

day routine pathology are considered, in the present situation only a subset is large and consistent enough to apply these rules rigorously (for example, testing sentinel nodes for tumour metastases, immunohistochemical panels for discriminating between different types of adenocarcinomas).^{26,27} A major risk for following this strategy is that by the time the process is completed, an alternative test emerges that is implemented in diagnostic pathology without going through this whole procedure. When we compare this with the situation of putting a new drug on the market, this is a remarkable situation, because the consequences of a substandard test can be as equally severe as the consequences of a substandard drug. If we want to develop clinical pathology as an evidence based branch of medicine, it is obvious that we should strive to extend our repertoire of validated tests.

Nevertheless, in many situations we have to provide answers without having such a validated test available, and we simply use the tools that are available. This can be acceptable as long as both pathologists and clinicians are aware of the limitations of the test used. In the case of obvious test results (for example, the CGH results of two tumours are 100% identical or 100% different) we can provide an answer that has a high degree of certainty, but in all other cases there is a poorly defined level of uncertainty, which should be considered in the ultimate diagnosis and communicated to the clinician who has asked for the test. Against this background, we have explored the possibilities of comparative genomic hybridisation for classifying multiple tumours within one patient as related or independent lesions. It is clear that CGH at this moment does not meet all the criteria described above for a validated diagnostic test—for example, the cut off for deciding whether or not tumours are related is not completely clear. This is further hampered by the fact that tumours may share identical chromosomal changes because they belong to the same histological category. For example, in squamous cell carcinomas loss of 3p, gain of 3q, and amplifications at 11q13 are relatively common, so that finding these changes in two different squamous cell carcinomas provides little information with respect to whether or not the tumours are related. In addition, it can be difficult to draw conclusions when there are relatively small numbers of chromosomal changes. It is more convincing when two tumours share 10 of a total of 20 chromosomal changes, rather than two of four different chromosomal changes. It is essential that we should look for standardisation of test interpretation, preferably based on clear data on the positive and negative predictive value of such tests. However, as with many other tests in pathology, these figures are not readily available, and we should be aware of this fact when interpreting these tests and reporting our conclusions. Nevertheless, when taking these limitations into account, the results of CGH could still contribute towards clinical decision making, as illustrated in the cases presented here.

“It is essential that we should look for standardisation of test interpretation, preferably based on clear data on the positive and negative predictive value of such tests”

When a second tumour presents in the same organ system as the first, the question invariably arises as to whether it is a recurrence or a second primary. The answer to this question may have clinical implications, both with respect to further diagnosis and treatment, and with respect to the patient, for whom a difference can sometimes be made between the presence of metastatic disease versus repeated successful treatment of primary malignancies. Morphological comparison and an extended immunophenotypic profile of different lesions can sometimes resolve the question, but the presence of a clonal mutation or a characteristic pattern of genetic alterations is the most direct way to establish a link between

Take home messages

- Comparative genetic hybridisation (CGH) patterns of gains and losses were useful in supporting the differentiation between metastasis or second primary, and in the identification of the primary tumour location in cases of metastases and two primary tumours in the seven patients analysed
- Thus, CGH is a very valuable diagnostic technique in patients with multiple tumours
- Microarray CGH has a higher resolution and sensitivity and data analysis is more straightforward than with chromosome based CGH, so that this technique will probably be widely used in routine pathology testing in the future

the two lesions, or in contrast, to suggest that the metachronous tumours represent independent events. For this purpose, the pathologist has the choice between testing for the presence of specific mutations (for example, the chance that two independent tumours in one patient share the same p53 mutation is very low),²⁸ using a panel of microsatellite markers,²⁹ or using CGH. Of these, CGH is the only one that tests on a genome wide scale. In our present study, chromosome based CGH has been applied. A new development in this respect is microarray based CGH, one of the “DNA chip” technologies. With this approach, chromosomes are no longer used as a template for hybridisation, but an array of microscopically small spots of genomic DNA is used instead. Every spot represents a unique DNA sequence for which the chromosomal locus is known.^{30,31} Microarray CGH has a higher resolution and sensitivity, and data analysis is more straightforward than with chromosome based CGH. It is to be expected that this technique will become widely used in routine pathology testing some time in the future.

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