

Technical report

Amplification of fluorescent in situ hybridisation signals in formalin fixed paraffin wax embedded sections of colon tumour using biotinylated tyramide

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Abstract

Fluorescent in situ hybridisation (FISH) is a powerful tool for the evaluation of chromosomal alterations in formalin fixed paraffin wax embedded sections of colorectal cancer. However, initial experiments using a two-step detection system for digoxigenin labelled chromosome specific centromeric probes resulted in a complete lack of hybridisation signal from a number of colorectal tumour sections. This was due to high levels of background autofluorescence observed in this tissue, which masked any relatively weak hybridisations present. To overcome this problem, a biotinylated tyramide mediated amplification system was incorporated into the FISH detection protocol. This involves the use of horseradish peroxidase to activate the biotinylated tyramide, resulting in the deposition of a large number of biotin molecules at the site of bound peroxidase, which corresponds directly to the location of hybridised probe. Final detection was by means of a streptavidin-FITC conjugate. Using this technique, a panel of 11 colorectal tumour samples studied to date have shown strong, specific hybridisation signals to the nucleus of tumour cells. Amplification of FISH signals by biotinylated tyramide has the potential to improve weak hybridisation signals in cells from numerous sources, using a variety of probe types, including single copy gene probes as well as centromere specific probes.

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Colorectal cancer is one of the most common forms of cancer, with approximately 210 000 new cases reported in Europe each year,¹ and 125 500 deaths due to this disease per year. It is the second most common malignancy in men and the third most prevalent in women. Due to the progression of colorectal tumours

through a normal-adenoma-carcinoma sequence, this tissue has proved ideal to study as a model of carcinogenesis. It is now thought that an accumulation of genetic alterations such as mutations or loss of chromosomal regions is necessary for transformation to a malignant phenotype, such alterations usually involving either oncogenes or tumour suppressor genes.² Investigations using techniques such as polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis, Southern blotting, and microsatellite analysis have identified numerous genetic changes in colorectal cancer including loss of heterozygosity of chromosomes 5q, 17p, and 18q.³⁻⁴ Loss of heterozygosity of chromosome 18q has been shown to affect prognosis in a group of patients with colorectal cancer,⁴ and it may be possible to identify other chromosomal regions that have such an effect by examining chromosome copy number within colorectal tumours.

Although methods such as microsatellite analysis and Southern blotting can be used to study genetic imbalances, these techniques do not preserve cellular architecture and samples contain a significant level of contamination by non-tumour tissue. In addition, they cannot pinpoint genetic changes to a particular cell or group of cells within a tumour. One technique that can overcome these problems is fluorescent in situ hybridisation (FISH).⁵ FISH has increased in importance over the past decade as a versatile and powerful method for detecting DNA target sequences in cells from a wide variety of sources, including formalin fixed paraffin wax embedded tumour samples. A variety of probes has been used for FISH, ranging from those that recognise centromeric regions of specific chromosomes to those that can identify single copy genes.

However, problems encountered using FISH include weak hybridisation signals in formalin fixed paraffin wax embedded material as well as the masking of hybridisation signal by autofluorescence of certain tissue types including adrenal, brain, and colon tissue,⁶ which

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may also be due in part to the effects of formalin fixation.⁷

Recently considerable progress has been made in developing new probe detection systems that can increase significantly the sensitivity of FISH, thus potentially overcoming these limitations. Of particular interest is the use of biotin labelled phenols, such as biotinylated tyramide, to amplify signals obtained in immunoassays.⁸ Further work^{9,10} demonstrated the applicability of this amplification procedure to a variety of immunodevelopment methods including western blotting and ELISA (enzyme linked immunosorbent assay). Since then, this technique has also been used in immunohistochemical investigations,¹¹ and more recently^{12,13} in *in situ* hybridisation.

We have successfully extended the tyramide technology to interphase cytogenetics of archival paraffin wax embedded colon tumours to overcome the problems of autofluorescence and weak signals, encountered when using conventional FISH development protocols.

Materials and methods

TISSUE SAMPLES

Samples of colon tumour were obtained from resected specimens submitted to the department of pathology, University of Aberdeen, for diagnostic purposes. Tissue samples had been fixed in 10% neutral buffered formalin for 24 hours at room temperature, routinely processed to paraffin wax and stored for up to six years at room temperature.

TISSUE PREPARATION

Sections 5 µm thick were cut onto APES coated glass slides, and heated to 65°C for up to 18 hours. The sections were then dewaxed by three successive 10 minute washes in xylene, followed by two, five minute washes in methanol. After air drying, the tissue sections were digested with 4 mg/ml porcine stomach mucosal pepsin (~3100 U/mg, Sigma, Poole, Dorset, UK) in 0.2 M HCl at 37°C for up to 30 minutes, rinsed in distilled water six times and post-fixed in Streck tissue fixative (Streck Laboratories, Omaha, Nebraska, USA) for 10 minutes. Sections were then dehydrated through a series of 70% and 100% ethanol washes for two three minute periods each, and dried at 55°C.¹⁴

IN SITU HYBRIDISATION

Probes specific for repetitive sequences at the centromeric region of chromosomes 3 (D3Z1), 9 (D9Z1), and 18 (D18Z1) were obtained from Appligene Oncor (Chester-Le-Street, Co Durham, UK), and were pre-labelled with digoxigenin. Before hybridisation, probes were diluted in a hybridisation mix consisting of 70% formamide, 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M Na citrate, pH 7.0), 500 mg/ml salmon sperm DNA, and 10% dextran sulphate. Diluted probe (20 µl) was applied to the tissue sections, and a coverslip was sealed on to the slide with rubber cement. Denaturation was then carried out at 85°C for five minutes, followed by hybridisation at 37°C for at least 16 hours using an OmniSlide thermal cyclor

(Hybaid Ltd, London, UK). After removal of the coverslips, sections were washed in 50% formamide/1× SSC then 2× SSC, each at 42°C for 20 minutes.¹⁵

PROBE DETECTION

Non-amplified detection

Slides were blocked with PN buffer (0.1 M NaPO₄ buffer pH 8.0) containing 0.5% blocking agent (Boehringer Mannheim, Lewes, East Sussex, UK) and 0.05% Tween 20 (PN-TB) for 15 minutes (all probe detection steps were carried out at room temperature). Sections were then sequentially incubated with sheep antidigoxigenin (1/200 dilution in PN-TB; Boehringer Mannheim) and FITC conjugated donkey antisheep (1/500 dilution in PN-TB; Stratech Scientific, Luton, Beds, UK) for one hour each, washing in PN-T (PN-TB minus the blocking agent) for 20 minutes after each antibody step. Sections were then counterstained with 5 µg/ml DAPI for 10 minutes, and washed in PN buffer for five minutes. Following dehydration in 70% and 100% ethanol, sections were mounted in Vectashield antifade (Vector Laboratories, Peterborough, UK).¹⁵

Biotinylated tyramide amplification

Slides were blocked with 0.05 M Tris-HCl, pH 7.6, containing 0.5% blocking agent and 0.05% Tween 20 (TB) for 15 minutes. The slides were then incubated with sheep antidigoxigenin (1/200 dilution in TB) for one hour, followed by three 10 minute washes in 0.05 M Tris-HCl, pH 7.6, containing 0.15 M NaCl (TBS). Next, a donkey antisheep biotin conjugate (Sigma) was applied to the sections (1/200 dilution in TBS containing 4% normal human serum) for one hour, and the sections washed for three 10 minute periods in TBS, and three five minute periods in 0.1 M Tris-HCl, pH 7.6, containing 0.15 M NaCl and 0.05% Tween 20 (TNT). A further blocking step was then carried out using 0.1 M Tris-HCl, pH 7.6, containing 0.5% DuPont blocking reagent (DuPont NEN, Hounslow, UK) and 0.15 M NaCl (TNB) for 30 minutes. Sections were then incubated with streptABCComplex/horseradish peroxidase (Dako, High Wycombe, Bucks, UK; prepared according to manufacturer's instructions) for 30 minutes, before being washed for three times five minutes in TNT. Biotinyl tyramide (DuPont NEN) was diluted 1/50 with 1× amplification buffer and applied to the slides for 10 minutes, before being washed off with TNT (three five minute periods). Finally, sections were incubated with FITC conjugated streptavidin (Dako; 1/100 dilution in TBS) for 30 minutes, washed for three five minute periods with TNT, and counterstained with DAPI as described previously.

CONTROLS

Both positive and negative controls were incorporated into each development protocol. Blood smears were used as positive controls, whereas negative controls consisted of slides hybridised with non-labelled probe, or sections incubated with TBS instead of primary antibody.

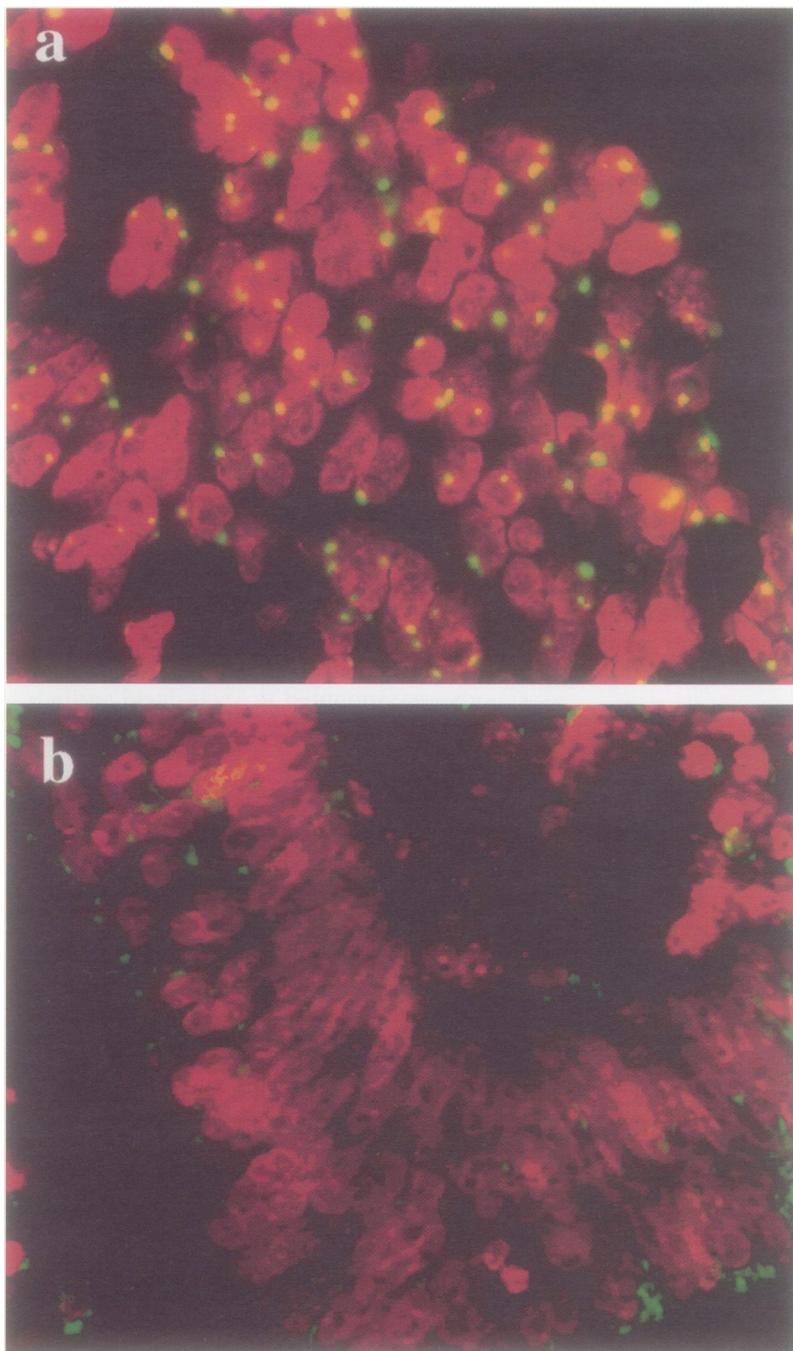


Figure 1 Analysis of chromosome 9 centromere in 5 µm sections of a formalin fixed paraffin wax embedded colorectal tumour by FISH. (a) Bright hybridisation spots are clearly evident in the nuclei of tumour cells developed using biotinylated tyramide amplification. (b) Non-amplified signal detection (sheep antidigoxigenin, FITC conjugated donkey antishoop) results in no visible hybridisation signals. Sections were counterstained with DAPI and pseudocoloured red using Quips SmartCapture FISH software to enhance visualisation.

MICROSCOPY

Sections were visualised using a Zeiss Axioplan II fluorescent microscope (Carl Zeiss, Welwyn Garden City, Herts, UK), and images were processed using QUIPS SmartCapture FISH software (Vysis UK, Richmond, Surrey, UK).

Results and discussion

While the use of FISH in cytogenetic studies has greatly improved our knowledge of chromosomal abnormalities arising in various pathological conditions, it is not without its limitations. One such limitation is the masking of relatively weak hybridisation signals by high levels of background autofluorescence. This

problem was encountered while carrying out FISH experiments on paraffin wax embedded colorectal tumour samples using a two-step detection protocol, which had previously been successful in a number of other tissues including breast.¹⁵ As can be seen in fig 1b, no detectable signal was observed when colorectal tissue sections were developed using this method. In addition, when viewed under the fluorescence microscope, the colorectal tissue was found to autofluoresce throughout all regions of the tumour. The complete lack of signal was not due to a technical failing as positive control slides produced visible hybridisation signals (results not shown).

Recently, a novel amplification system for immunodevelopment techniques was described,⁸ which involves the use of biotinylated tyramide. It is thought that the presence of an initiating enzyme such as horseradish peroxidase (HRP) in the development protocol catalyses oxidation of the biotinylated tyramide forming free radicals, which then react with electron rich moieties on certain amino acid residues on bound proteins.⁸ This leads to the deposition of many biotin molecules at or around the site of bound HRP. Due to the short lived nature of such free radicals, this reaction only occurs in close proximity to the site of formation, which prevents the signal becoming diffuse. The final stage in the procedure utilises the strong affinity between biotin and avidin (conjugated to a reporter molecule), leading to enhanced levels of reporter molecule being bound at sites of interest.

This amplification was incorporated into a FISH development protocol for paraffin wax embedded colorectal samples using centromeric probes specific for chromosomes 3, 9, and 18. As can be seen in fig 1a, this resulted in strong hybridisation signals throughout the tumour section, without apparent loss of resolution. In addition, no FITC signals were observed in negative control sections, ruling out the possibility of non-specific false positive results.

This study demonstrates that by applying a relatively simple amplification system when developing FISH experiments, it is possible to obtain easily identifiable hybridisation signals from previously problematic tissue.

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