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Quantitative multiplex quantum dot in-situ hybridisation based gene expression profiling in tissue microarrays identifies prognostic genes in acute myeloid leukaemia

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ABSTRACT

Measurement and validation of microarray gene signatures in routine clinical samples is problematic and a rate limiting step in translational research. In order to facilitate measurement of microarray identified gene signatures in routine clinical tissue a novel method combining quantum dot based oligonucleotide *in situ* hybridisation (QD-ISH) and post-hybridisation spectral image analysis was used for multiplex *in-situ* transcript detection in archival bone marrow trephine samples from patients with acute myeloid leukaemia (AML). Tissue-microarrays were prepared into which white cell pellets were spiked as a standard. Tissue microarrays were made using routinely processed bone marrow trephines from 242 patients with AML. QD-ISH was performed for six candidate prognostic genes using triplex QD-ISH for *DNMT1, DNMT3A, DNMT3B,* and for *HOXA4, HOXA9, Meis1*. Scrambled oligonucleotides were used to correct for background staining followed by normalisation of expression against the expression values for the white cell pellet standard. Survival analysis demonstrated that low expression of *HOXA4* was associated with poorer overall survival ($p = 0.009$), whilst high expression of *HOXA9* ($p < 0.0001$), *Meis1* ($p = 0.005$) and *DNMT3A* ($p = 0.04$) were associated with early treatment failure. These results demonstrate application of a standardised, quantitative multiplex QD-ISH method for identification of prognostic markers in formalin-fixed paraffin-embedded clinical samples, facilitating measurement of gene expression signatures in routine clinical samples.

1. Introduction

Diagnosis of cancer typically relies on morphological appearances and immunohistochemistry (IHC), together, in some cancers, with cytogenetic and molecular analyses. However, in the emerging era of tailored therapy there is a growing need for new predictive biomarkers. Global gene expression profiling using microarrays has been used to identify such markers. However, though expression microarrays are ideal for discovery their use is limited by lack of morphological correlation, a semi-quantitative nature and requirement for high quality fresh-frozen tissues, most routinely processed biopsy samples being in the form of formalin-fixed and paraffin-embedded tissue (FFPET) [1]. A combined approach enabling quantitative measurement of the expression of multiple genes together with morphological assessment in fixed tissue samples would overcome the above problems and provide a tool for investigation of the expression patterns and roles of microarray identified genes in cancer.

In situ hybridisation (ISH) and IHC can be used for *in situ* visualisation of mRNA and protein, respectively in routinely processed FFPET sections [2]. Whilst IHC is limited by the availability of antibodies, probes for ISH can easily be constructed for any gene of interest [2,3]. However, ISH has been compromised by poor signal-to-noise ratio due to low fluorescence efficiency of fluorescent markers and high autofluorescence of paraffin embedded tissues [4–7]. Additionally, neither ISH nor IHC are quantitative in most studies.

We have previously demonstrated the utility of a novel quantum dot (QD) based ISH (QD-ISH) method in FFPET [8,9]. In order to facilitate translation of this approach to clinical research and diagnostics, this method requires capacity for high-throughput, and robust quantitation and standardisation to correct for inter-experimental and inter-laboratory variations. Jubb et al. [10] were the first to use standards for quantitative analysis of tumor markers identified by IHC and ISH in tissue microarrays (TMAs),

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embedding sense and anti-sense RNA standards into agarose. However, RNA strips do not fully recapitulate tissue as their hybridisation kinetics are likely to differ due to reasons of mRNA fixation in tissue and the presence of surface irregularity after tissue sectioning, both of which may result in different hybridisation efficiency. For this reason we used cell pellets embedded into agarose gel as standards across TMAs instead, the cellular nature of which more accurately reflects the hybridisation dynamics than does a synthetically prepared RNA sample. As previously described, we applied triplex QD-ISH and spectral imaging for data capture and analysis [9] and developed an equation for normalisation and standardisation of expression values. In the present study this method was applied to a large cohort of patients with acute myeloid leukaemia (AML), a genetically heterogeneous disease for which new prognostic molecular markers are needed.

Here we describe a novel method of quantitative expression measurement of multiple genes in routinely processed FFPET bone marrow trephines using multiplex QD-ISH in tissue microarrays, which allowed us to analyse a large number of patient samples.

2. Materials and methods

2.1. Sample selection

Presentation bone marrow trephine samples from 242 patients diagnosed with AML between 1994 and 2005 were retrieved from the histopathology archives at Manchester Royal Infirmary. All trephine biopsy samples were routinely processed, formalin-fixed, paraffin-embedded and EDTA decalcified at presentation. All material used was residual diagnostic tissue, anonymised and consent for its use in research granted.

2.2. Preparation of standard

For this, peripheral blood white cells were used. Thirty milliliters of peripheral blood was obtained prior to a peripheral blood haemopoietic progenitor cell collection. The donor had received 10 μg/kg granulocyte stimulating growth factor (Lenograstim; Chugai, London, UK) for 5 days prior to collection. Peripheral blood was poured into Ficoll (Pharmacia, St Albans, UK) and centrifuged at 2000 rpm for 20 min. The mononuclear layer was removed, washed with normal saline and fixed in 4% (v/v) formaldehyde/PBS (Genta Medical, York, UK). White cell pellets were then embedded in agar (Agar Scientific, Essex, UK) followed by embedding in paraffin wax using standard protocols.

2.3. TMA construction

Tissue microarrays were prepared using an ATA 100 tissue array machine (Chemicon International, Temecula, CA, USA). For this, cylindrical cores 1.5 mm in diameter were taken from paraffin embedded bone marrow trephine samples using a hollow needle and inserted into a precisely spaced 'recipient' paraffin wax block. Cores were obtained from areas showing leukaemic infiltration on H&E staining.

Each TMA contained between 20 and 24 different patient samples. From each patient sample three cores were removed and placed at different sites of the TMA block to maximise separation between related cores within a TMA. Similarly, three cores of the pre-prepared cell pellets were embedded in each TMA. A total of fourteen TMAs were prepared. Sections were cut from the TMA blocks and mounted onto coated glass slides; using four micron thick sections for H&E staining and seven micron sections for ISH. Each core from each patient contained at least 20% leukaemic blasts and was representative of the whole trephine sample.

2.4. In situ hybridisation

A total of six genes from two gene families were studied: *HOXA4*, *HOXA9* and *Meis1* from the homeobox gene family, and *DNMT1, DNMT3A* and *DNMT3B* from the DNA-methyl transferases. ABL and beta 2 microglobulin were used as housekeeping genes to evaluate sample viability; ABL is a well established housekeeping gene and used widely in gene expression analysis in acute leukaemia. Anti-sense cDNA oligonucleotide probe sequences (50-mer) specific to the above genes and their corresponding scramble probes were used (anti-sense probe sequences are shown in Table 1). The probes were HPLC purified and biotin modified via a TEG spacer at the 3′end (Eurogentec, Seraing, Belgium). Probes targeting the genes from each gene family were conjugated to three different quantum dots (QDs) (605, 655, 705 nm) per family. Triplex QD-ISH for each family was performed on 7 μm thick sections for both anti-sense and scramble probes. QD-ISH for the control genes was performed in duplex. Probe design and QD-ISH was performed as previously detailed in Tholouli et al. [9].

2.5. Imaging analysis

Up to four images per core sample were captured at 400× using a Leitz Diaplan fluorescence microscope (Leitz, Germany) with a 490 nm excitation long pass filter and a CRI Nuance spectral analyzer (Cambridge Research and Instrumentation Inc., Woburn, USA) [9]. Images were collected using a standard exposure time of 1000 ms at 5 nm wavelength intervals from 450 to 720 nm creating a 3D optical profile (cube) with the dimensions *x*, *y* and wavelength, containing the complete spectral information for every pixel at each wavelength. Using the spectra for autofluorescence and that of the relevant QDs, spectral unmixing allowed digital separation of this information [11].

2.6. Signal quantitation

The resultant unmixed images were then transferred to IPLab (Scanalytics, MD, USA) for measurement of fluorescent signal

Table 1
Reverse complement sequences of anti-sense probes.

Gene	Reverse complement oligonucleotide sequence
ABL	5′-ACTCAGACCCTGAGGCTCAAAGTCAGATGCTACTGGCCGCTGAAGGGCTT-3′
β2M	5′-CCAGAAAGAGAGAGTAGCGCGAGCACAGCTAAGGCCACGGAGCGAGACAT-3′
HOXA9	5′-CCGCTTTTTCCGAGTGGAGCGCGCATGAAGCCAGTTGGCTGCTGGGTTAT-3′
HOXA4	5′-CTCGAAGGGAGGGAACTTGGGCTCGATGTAGTTGGAGTTTATCAAAAACG-3′
Meis1	5′-CCTCCATGCCCATATTCATGCCCATTCCACTCATAGGTCCTGGTGCTCTA-3′
DNMT1	5′-GCCAGGTAGCCCTCCTCGGATAATTCTTCTTTACGTAATTTGGTTTCCAA-3′
DNMT3A	5′-TTCTCAACACACACCACTGAGAATTTGCCGTCTCCGAACCACATGACCCA-3′
DNMT3B	5′-AGTTTGTCTGCAGAGACCTCGGAGAACTTGCCATCGCCAAACCACTGGAC-3′

β2 M = β2 microglobulin.