University of Dundee

Development and optimisation of immunostaining methodology for the investigation of p53 isoforms.

Thineskrishna Anbarasan

Supervisor: Dr Jean-Christophe Bourdon

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Background

TP53 is the most frequently mutated gene in cancer. In response to stress stimuli including DNA damage response and oncogene activation, p53 mediates a complex transcriptional program. This involves a plethora of biological responses ranging from apoptosis, cell senescence and cell cycle arrest. p53 is also involved in other non-canonical processes such as metabolic regulation and pluripotency (Kastenhuber & Lowe, 2017) . It is therefore unclear how a single protein such as p53 is able to mediate such a vast network of biological activities. The counterparts of p53, its isoforms, are increasingly found to be involved in the responses of full-length p53 (FLp53 or p53α), being able to both inhibit and complement p53's actions in a cellular-context dependent manner. Till date twelve p53 isoforms (p53α, p53b, p53g, Δ40p53α, Δ40p53β, Δ40p53γ, Δ133p53α, Δ133p53β, Δ133p53γ, Δ160p53α, Δ160p53β and Δ160p53γ), depicted in **Figure 1**, have been characterised from nine distinct TP53 mRNA transcripts. The expression of p53 isoforms have been correlated with cancer progression and survival outcomes in various cancer types illustrating their potential as a biomarker (Joruiz & Bourdon, 2016). Attempts to identify p53 isoforms in situ via conventional immunostaining have been faced with challenges given they are not as abundantly expressed as structural proteins such as actin, vimentin and cytokeratin.

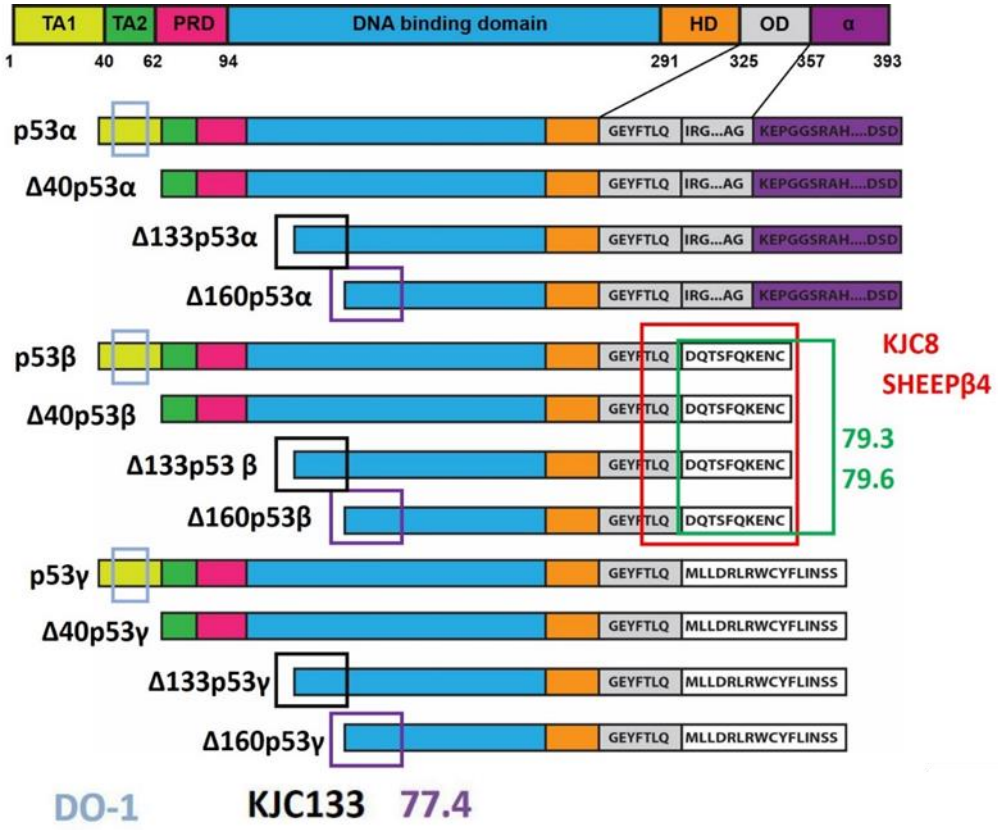

Figure 1: Functional domains of p53 isoforms are presented with respect to the that of p53α (corresponding colours). The β and γ domains are both uncoloured as they are not present in p53α. The epitopes of the various antibodies (DO-1, KJC133, 77.4, 79.3, 79.6, Sheepβ4 and KJC8) to be detailed in Table 2 are demarcated with separate colours.

Aims

1. Evaluate if staining observed with tyramide signal amplified immunohistochemistry is sensitive to target p53 isoforms?

2. Determine if the use of tyramide signal amplified immunohistochemistry enhance detection of p53 isoforms in FFPE cells/tissue.

Methods

Biotin-tyramine (tyramide) signal amplification was employed to detect p53 isoforms in situ via immunohistochemistry using fresh-formalin fixed paraffin embedded (FFPE) tissue and cell sections. A schematic outlining the basis of tyramide immunohistochemistry (TIHC) and tyramide immunofluorescence (TIF) is illustrated in **Figure 2**.

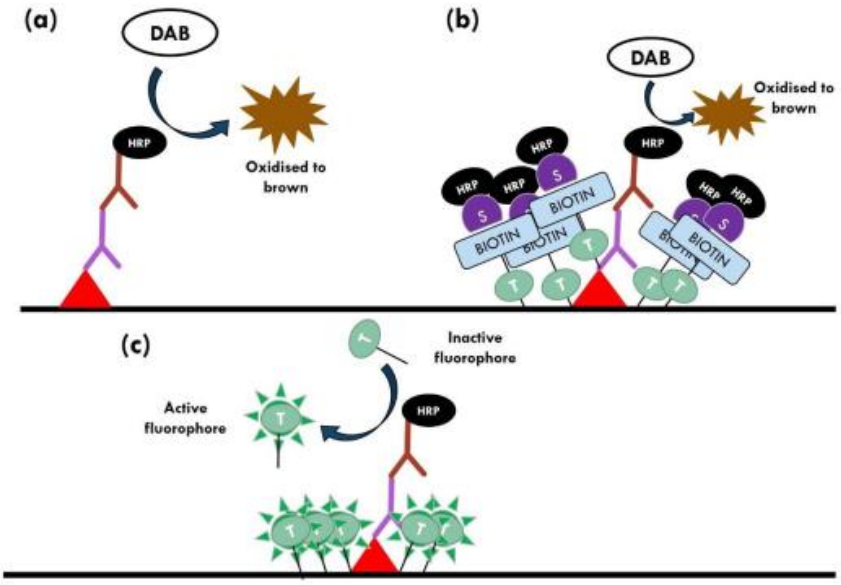

Figure 2: Schematic of tyramine immunostaining system. **(a)** Conventional IHC with DAB reaction catalysed by HRP conjugated to the secondary antibody. **(b)** Signal amplification with biotinylated tyramine. **(c)** Tyramine conjugated to a fluorophore activated in the presence of HRP and subsequently deposited at the site of antigen.

H1299 cells transfected with individual p53 isoforms were developed into FFPE blocks following confirmation of transfection success using western blot. FFPE sections were utilised to develop the immunohistochemistry protocol to detect p53 isoforms using tyramide signal amplification. An overview of the methodology employed is illustrated in **Figure 3**. Protocol optimisation involved altering individual components of the immunohistochemistry protocol to reduce non-specific background staining. Optimised protocol was subsequently adapted to immunofluorescence to determine localisation of p53 isoforms.

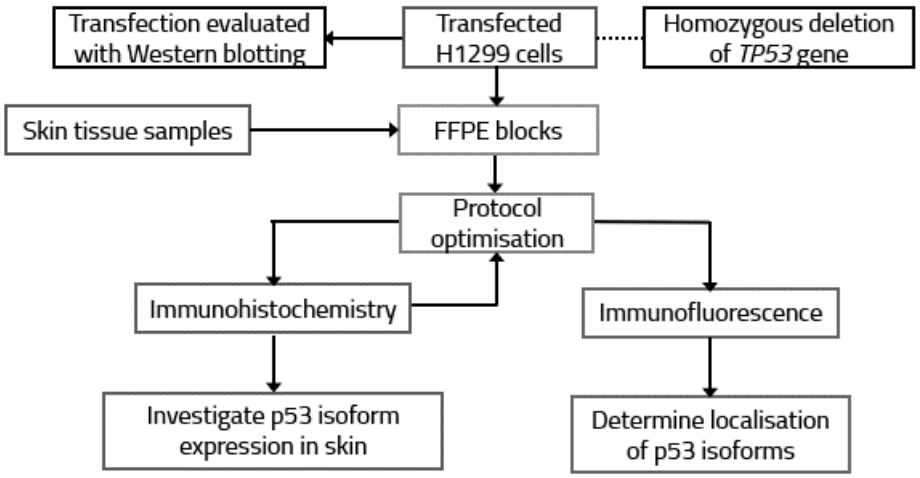

Figure 3: Project schematic.

Tissue specimen used in this study are detailed in **Table 1.**

S/N	Tissue Description	Source
1	Healthy breast skin removed during breast reduction surgery from 31 year-old-female	Tayside Tissue Bank
2	Poorly differentiated cutaneous squamous cell carcinoma of the right temple from an 83-year-old male.	Tayside Tissue Bank

Results and Discussion

Tyramide IHC results in increased background staining

Negative control stains (primary antibody step omitted) performed on tissue (not shown here), the use of TIHC correlated with a higher background signal. A predisposition for tyramide to form dimers with the extracellular matrix (ECM) has previously been reported (Volante, Pecchioni & Bussolati, 2000). Therefore, to determine if the source of background signal we observed was related to unblocked antigenic receptors (i.e. macromolecules present in the ECM) native only to tissue, we performed TIHC staining of FFPE transiently transfected H1299 cells (**Figure 4**). The expression of the target isoform by the cells was confirmed via immunoblotting. Although there was evidence of specific staining of H1299 cells expressing the target isoform on visual inspection, there was a significantly high background signal. This rendered the transfected cells largely indiscernible. Together, these results suggest our TIHC protocol is not optimised and there is gross amplification of a non-genuine signal which is consistently observed even in an ECM void environment.

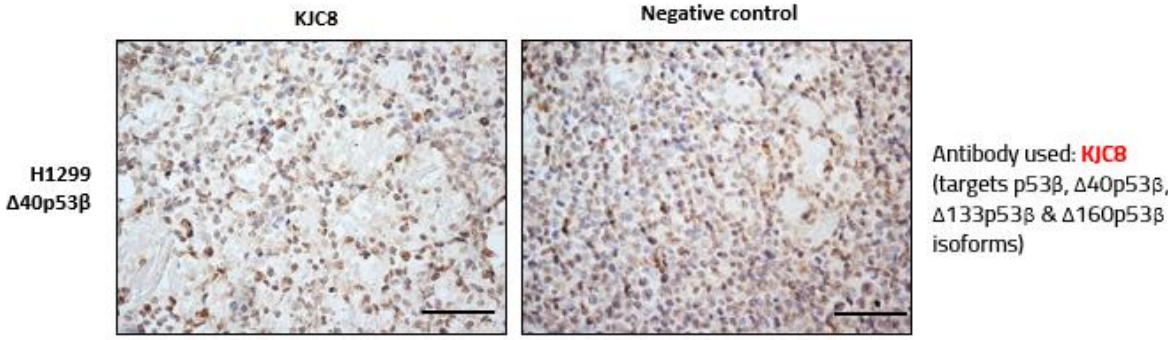

Figure 4: TIHC staining of FFPE Δ40p53β transfected H1299 cells with KJC8. High density background signal observed in negative control which renders the antibody specific staining undistinguishable.

Optimised Tyramide IHC protocol

To investigate the effect of altering immunostaining protocol variables on the background signal, we designed a negative control IHC assay (not shown here). The following changes as described in **Table 3** were incorporated to the final protocol based on respective observations from the negative control assay.

Step	Modification	Reason
Endogenous peroxidase blocking	Changed 0.3% H_2O_2/PBS to 3.0% H_2O_2/PBS	There was a slight decrease in background signal on semi-quantification
Protein blocking	Changed blocking medium from FCS to 5% BSA/PBS	Significantly reduced background signal due on negative control assay
1° antibody diluent	Changed 1° antibody diluent from FCS to 5% BSA/PBS	Significantly reduced background signal due on negative control assay
Repeat avidin/biotin blocking	Added avidin and biotin blocking step after protein blocking	We hypothesised that protein blocking step may introduce biotin contributing to background signal (Not formally tested)
Streptavidin-HRP concentration	Reduced streptavidin-HRP concentration from 0.5µg/ml to 0.3µg/ml	Reduces non-specific HRP deposition contributing to background signal.
DAB exposure time	Reduced DAB exposure time from 180s to 150s	Reduces deposition of non-specific brown stain.
Haematoxylin staining	Increased from 5s to 10s	Improves contrast of nuclei against amplified signal.

Table 3: Modifications made to TIHC protocol.

The optimised TIHC protocol when utilised to stain FFPE transfected H1299 cell sections, resulted in a visibly significant lower background signal on negative controls (**Figure 5**). When stained with antibodies targeting p53 isoforms (Del133, 79.3, KJC8), cells which were successfully transfected and expressing the isoform of interest were discernible.

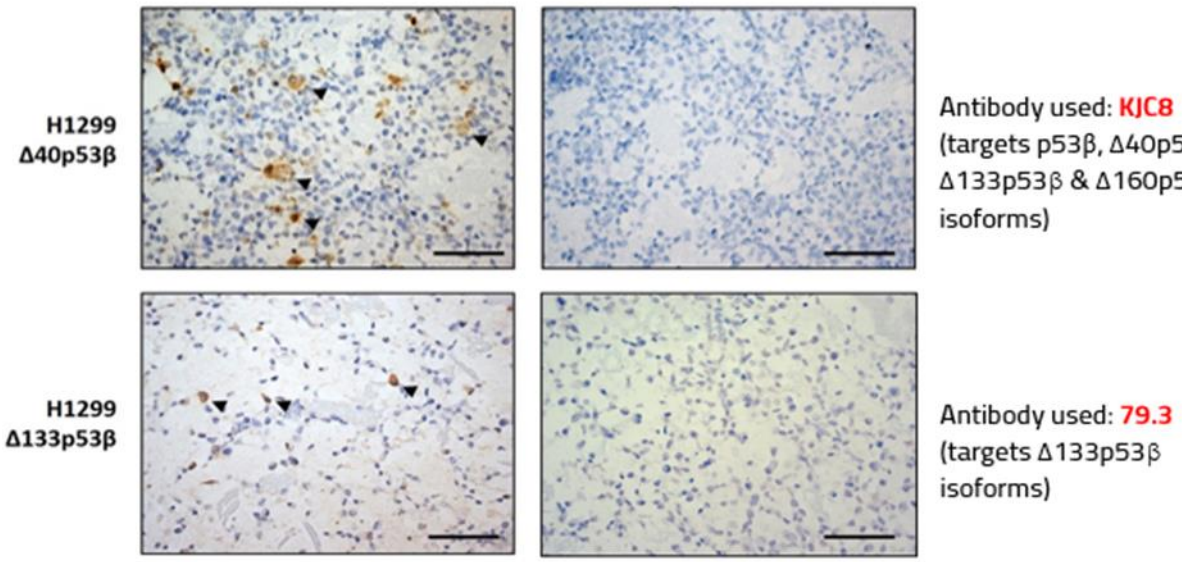

Figure 5: Staining of FFPE H1299 transfected cells with optimised protocol. (Top row) Δ40p53β transfected cells stained with KJC8. (Bottom row) Δ133p53β transfected cells stained with 79.3. Black arrows indicate successfully transfected cells expressing the target isoforms. Image objective: 20x. Scale bar: 100µm

Immunofluorescence investigation of p53 isoforms

To discern the localisation of the p53 isoforms we adapted the TIHC protocol to immunofluorescence. Here, we stained FFPE transfected H1299 cells and stained them with antibodies (KJCA133, KJC8 and 79.3) to determine the subcellular localisation of Δ133p53α, Δ133p53β and Δ40p53β (**Figure 6**).

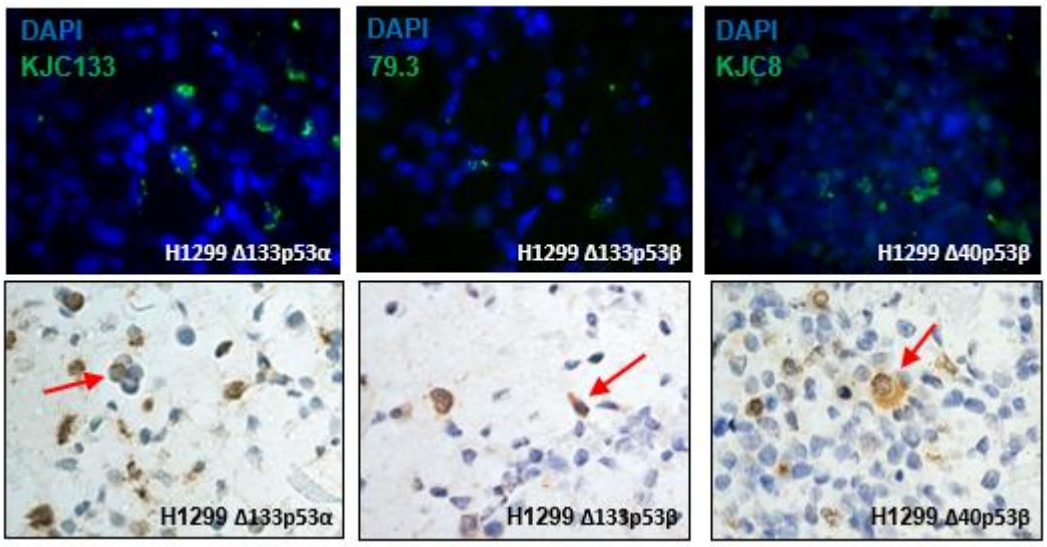

Figure 6: Localisation of p53 isoforms with tyramide immunofluorescence of transfected H1299 cells. On staining Δ133p53α transfected H1299 cells with KJCA133, a perinuclear localisation was observed. On staining Δ133p53β transfected H1299 cells, foci within the nuclei are observed. Staining of Δ40p53β transfected H1299 cells with KJC8 revealed a cytoplasmic localisation.

Preliminary investigation of p53 isoform expression in human tissue

To determine the expression of endogenous p53 isoforms in healthy breast skin, we performed TIHC, using the optimised protocol (**Figure 7**).

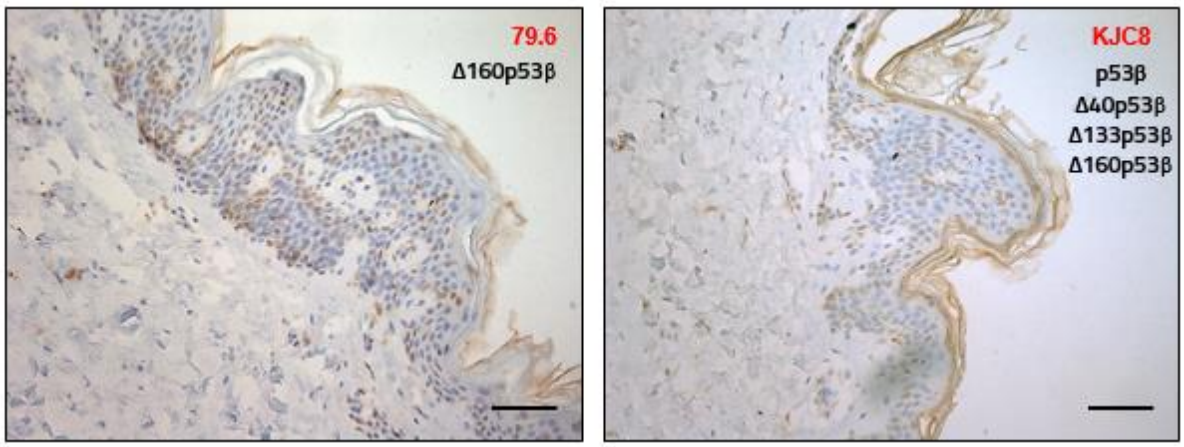

Figure 7: TIHC staining of FFPE normal breast skin with antibodies against p53 isoforms.

Here we investigate the expression of p53 isoforms in FFPE section of a poorly differentiated cutaneous squamous cell carcinoma (cSCC) of the right temple resected from an 83-year-old male. We stained these sections with the following panel of antibodies (KJC8, 79.3, 79.6 and KJC133) particularly focussing on the β isoforms of p53. A characteristic pattern of staining was observed with KJC8 (not shown here). To confirm the staining pattern observed with KJC8 using TIHC, we performed tyramide immunofluorescence to localise the isoforms stained (**Figure 8**). The staining observed with KJC8 is coherent with the stratum granulosum which decreases in intensity with progression towards the core of the cSCC. At the peri-tumoral region where the BM was intact, expression of KJC8 at the stratum granulosum was more intense compared to the area with BM invasion.

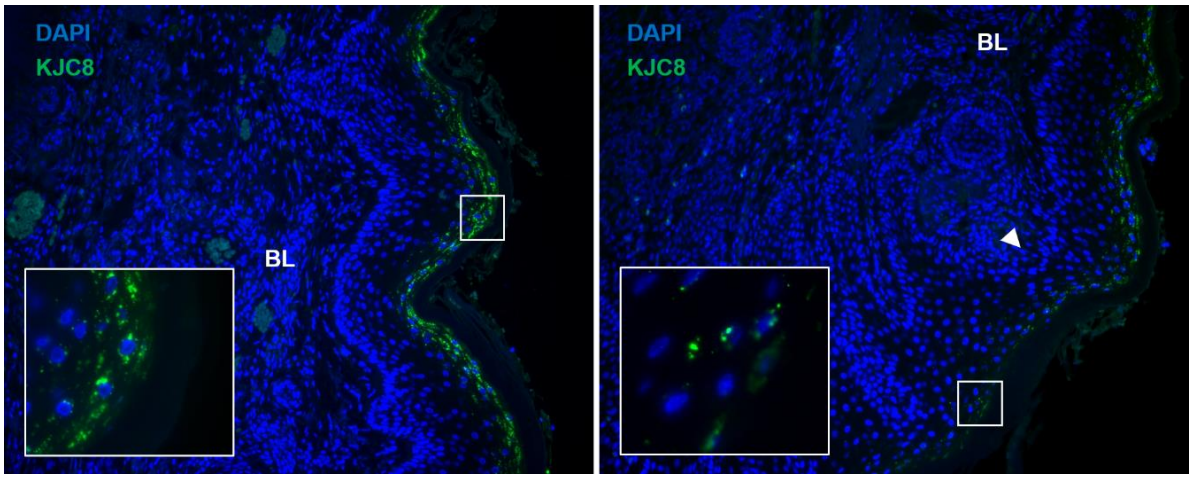

Figure 8: Tyramide immunofluorescence of cSCC with KJC8. A decrease in KJC8 staining specific to β isoforms of p53 is consistently observed adjacent to the site of basement membrane (BL) invasion (white arrow). The stained isoforms are localised to the cytoplasm with perinuclear aggregates.

Conclusion

In this study we have showed the detection of p53 isoforms can be enhanced with the use of biotin-tyramine signal amplification system. Additionally, the developed protocol can be applied to immunofluorescence which has revealed interesting preliminary insights into the role of p53 isoforms in skin cancer. The application of this protocol to other cancer types would aid deciphering the role of p53 isoforms in tumorigenesis.

References

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