Evaluation of the relationship between tumour LINE1 expression status and immune microenvironment in the context of hepatocellular carcinoma

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1 ACKNOWLEDGEMENTS

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2 INTRODUCTION

2.1 Hepatocellular carcinoma causes a significant burden of disease

Hepatocellular carcinoma (HCC) is the commonest form of liver cancer, and a leading cause of cancer-related deaths worldwide, with increasing incidence[1, 2].

Even when diagnosed early and treated with curative intent, HCC frequently recurs[2, 3]. Increasing disease burden and lack of treatment options have created a requirement for new therapeutic options.

2.2 The immune system can influence tumour progression

A recognised balance exists in cancer between activation of the immune system (immunostimulation), and suppression of the immune system (immunosuppression)[4].

Tumour infiltrating lymphocytes (TILs), including CD4+ and CD8+ lymphocytes, are attracted by tumour antigens and cytokines[7]. Specific subsets of TILs impact prognosis[7]. Both CD4+ and CD8+ lymphocytes are required for an effective anti-tumour response. High levels of CD8+ cells stimulate tumour death, and CD4+ cells are required for activation of these CD8+ cells[6]. M2 macrophages also infiltrate tumours; these express CD163 and have a pro-tumorigenic response[5].

2.3 Distinct immune subclasses have been identified in HCC

Analysis of HCC tumour cells, surrounding immune cells, and stroma identified inflammatory changes in 24% of cases[10]. These cases had higher expression of immune cells, thus were said to belong to an 'immune class'[10]. These signatures also supported potential responsiveness to immunotherapies[10]. Patients without expression of these immune signatures belong to a 'non-immune' class of HCC.

Subclassification was performed based on stromal activation status: nonactive stroma classified patients as belonging to an 'Active Immune' subgroup, whilst active stroma denoted an 'Exhaustive

Immune' subgroup[10]. The Exhaustive Immune subgroup had worse prognosis than the Active Immune subgroup[10].

2.3.1 Immunotherapies are targeted treatments which have dramatically improved prognosis in other cancers

Immunotherapies regulate T-cell pathways and target immune checkpoints, triggering the patient's immune system to attack the tumour[10]. Dendritic cells express immune checkpoints CTLA-4 and PD-1, which are targets of immunotherapies[11]. Trials into the use of immunotherapies in HCC are underway; however, understanding the immune microenvironment in HCC is critical to determine which patients will respond to treatment[10].

2.4 LINE1 RETROTRANSPOSONS ARE WELL-RECOGNISED IN HCC

Long Interspersed Nuclear Element 1 (LINE1) retrotransposons are mobile genetic elements, making up 17.5% of the genome[12]. These elements utilise a 'copy-and-paste' mechanism to retrotranspose to different genomic locations[12]. Hypomethylation of the LINE1 promoter activates these retrotransposons, triggering insertional mutagenesis which alters gene structure and function[12]. LINE1 dysregulation and subsequent mutagenesis is well-recognised in HCC[13]. Whilst LINE1 mobilisation occurs in a minority of HCC tumours, it adds to the wider collection of mutations which must occur for tumorigenesis[12].

Transcriptome analysis of other GI cancers found LINE1 retrotransposition inversely correlated with expression of immune regulatory genes[14]. This relationship has not been previously demonstrated in HCC.

2.5 Transforming growth factor beta (TGF-β) is a signalling factor in the immune microenvironment

The TGF-β signalling pathway is initiated by binding of TGF-β ligands to receptors, resulting in phosphorylation of SMAD proteins which regulate transcription[15]. 40% of HCC has TGF-β signalling

mutations, and elevated TGF-β induces immunosuppression and correlates with poor prognosis[15, 16]. Transcriptome data revealed three groups of HCC based on TGF-β signalling status: normal (same expression of TGF-β as surrounding non-tumour tissue), active (greater expression than non-tumour tissue), and inactive (reduced expression compared to non-tumour tissue)[16]. Unpublished RNAseq analysis by Shukla et al. laboratory group showed reduction in TGF-β signalling in LINE1 knockdown cells.

3 METHODS

3.1 RNA-sequencing analysis

HCC patient RNA-sequencing data from The Cancer Genome Atlas (TCGA) was analysed for transcript expression of LINE1, pSMAD3, and TGF-β1. Expression is displayed as FPKM RNAseq read counts. Sia et al. provided immune classification of TCGA data[10]. Chen et al. provided TGF-β classification[16].

3.2 Immunohistochemistry

3.2.1 Ventana

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections using the automated Ventana Discovery XT system (Ventana Medical Systems, Inc.). Manufacturer's recommendations were followed.

Anti-CD4, -CD8, and -CD163 rabbit primary antibodies (VMSI), anti-pSMAD3 (Santa Cruz Biotechnology, Inc.), and anti-LINE1 (Millipore) antibody were optimised for HCC tissue. Optimal tissue staining was achieved by primary antibody titration.

Secondary antibody was applied for 16 minutes. Primary antibody visualisation was performed using non-species specific Discovery ChromoMap diaminobenzidine (DAB) kit (VMSI). Nuclei counterstaining was performed with haematoxylin (VMSI), enhanced by blueing reagent (VMSI). Slides were hand-mounted with DPX and scanned using Aperio CS2 (Leica Biosystems).

3.2.2 Cell line construction

To create paraffin-embedded cell pellets for IHC, cells were grown in 75cm^2 flasks to 90% confluency. Cells were washed with PBS, 4% formalin was added, then cells were removed by scraping and left for 2 hours in formalin. Cells were centrifuged, supernatant was removed and cells were washed. Cell pellets were resuspended and quickly centrifuged to create cell pellets encased in Histogel Specimen Processing Gel (ThermoFisher), which were placed in tissue cassettes for

processing and paraffin embedding. 5µM slices of cell blocks were cut and mounted onto slides for staining.

3.3 Cell culture

HUH7 human HCC cells were cultured in T75 flasks in RPMI-1640 medium, HEPES modification (Sigma-Aldrich), with added 10% Foetal Bovine Serum (Gibco Life Technologies), 1% L-glutamine (Gibco Life Technologies), and 1% penicillin/streptomycin (Gibco Life Technologies).

Four HUH7 cell lines were cultured: wild type control (WT), two LINE1-knockdown lines (p128 and p13), and a non-target control (NT). WT HUH7 cell line had been modified previously by the Shukla laboratory via shRNA knockdown to create two cell lines with downregulated LINE1. Cell culture was performed in a Class II hood. Cells were inspected by light microscopy prior to subculturing to determine confluency, ensure HUH7 phenotype, and check for contamination. Cells were passaged at 90% confluency. Cells were subcultured by removing existing media and rinsing with PBS, before adding 1% trypsin (Sigma-Aldrich) for 5 minutes to detach cells. Media was added to neutralise the reaction. The cell suspension was centrifuged at 500xg for 3 minutes, liquid was aspirated off, and the cell pellet was re-suspended in fresh medium. Cells were incubated at 37°C with 5% CO_2.

3.4 Statistical analysis

All data is presented as mean ± standard error of the mean. GraphPad © Prism 6 software was used for statistical analyses, and a P value less than 0.05 was considered significant.

4 RESULTS

4.1 Characterisation of LINE1 transcript expression by immune activity

The initial objective of this project was to explore any association between LINE1 expression and HCC immune microenvironment.

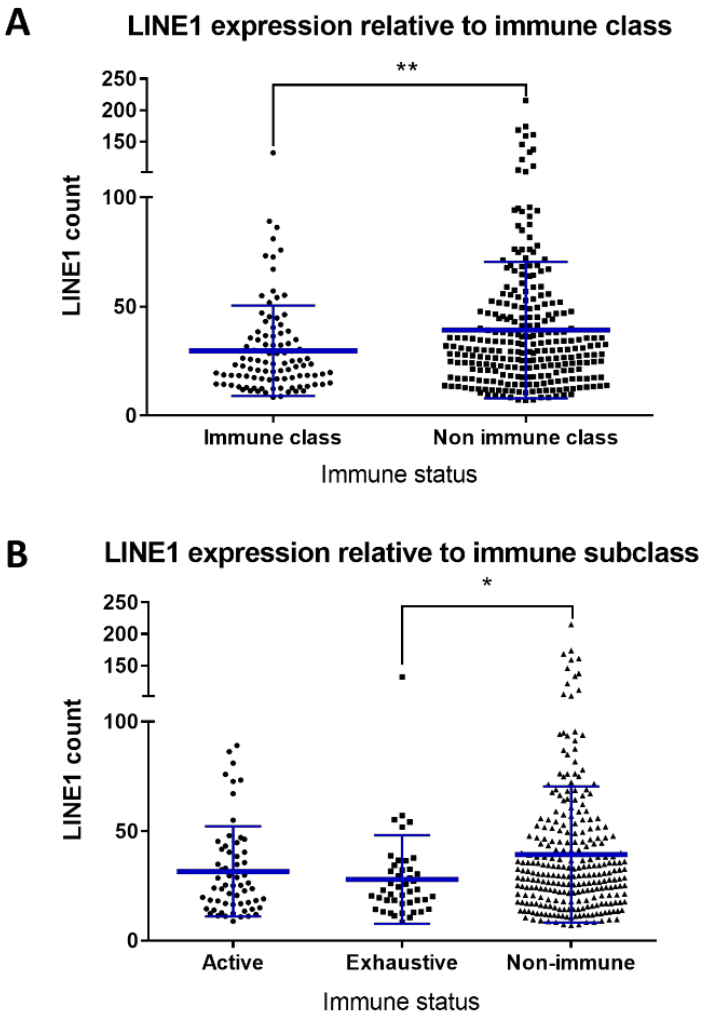

Figure 1: Graphs showing RNAseq results from 372 patients. (A) LINE1 expression categorised into immune and non-immune classes of HCC (Mann-Whitney U test, P=0.0037). In (B), the immune class has been further classified into active and exhaustive immune subclasses (Kruskal-Wallis test, P=0.0146).

One-way ANOVA analysis demonstrated significant variations in LINE1 expression based on immune status. LINE1 expression was significantly higher in the non-immune class of HCC than the immune class. LINE1 expression was significantly higher in the non-immune class than the exhaustive immune subclass.

4.2 Immunohistochemistry

4.2.1 Immunohistochemistry showed variations in tumour LINE1 expression status in HCC

TMAs were initially stained to identify tumour LINE1 expression status, to allow for subsequent analysis of immune activity relative to LINE1 expression.

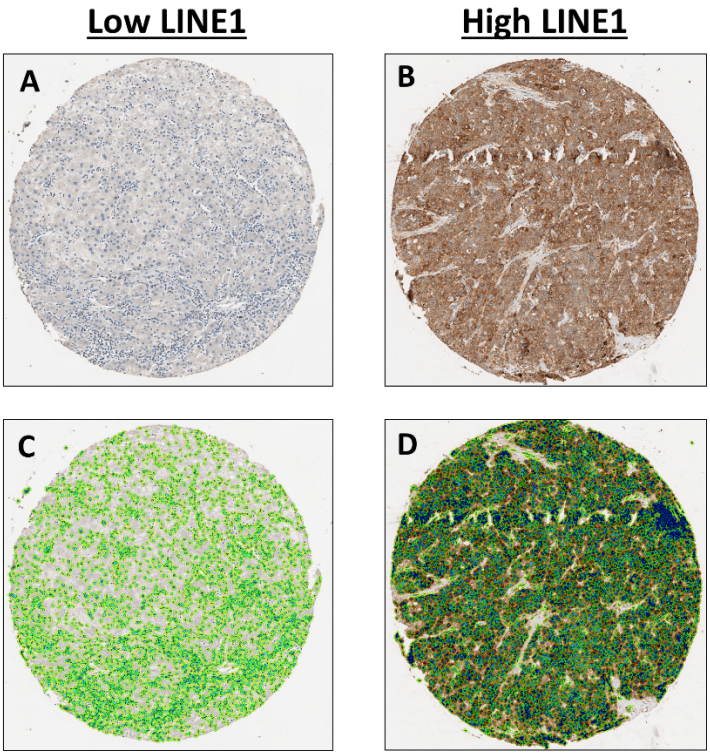

Figure 2: Immunohistochemistry of LINE1 demonstrated variations in LINE1 expression. This figure shows two samples, one with low LINE1 expression (A, C), and one with high expression (B, D). C and D demonstrate use of the algorithm, which was used to calculate LINE1 positivity.

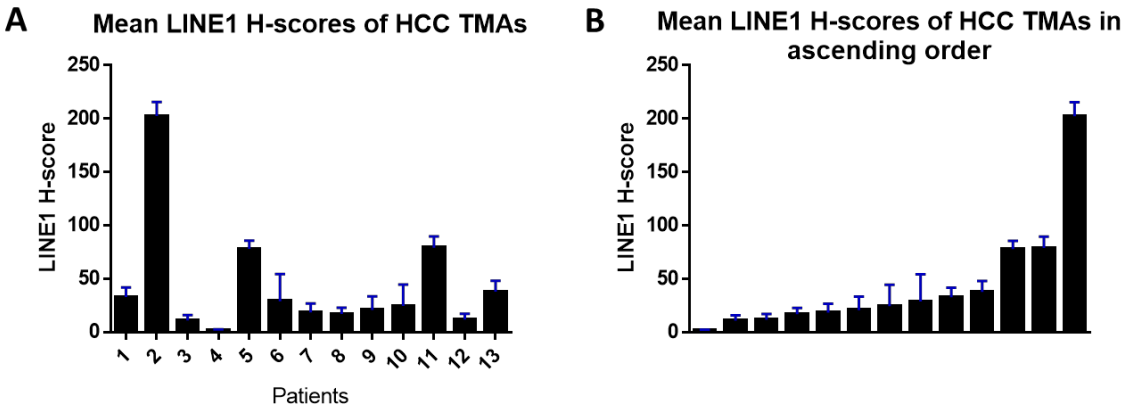

Figure 3: Graphs illustrating LINE1 cytoplasmic H-scores, as determined by IHC performed on HCC TMAs. (A) Mean LINE1 H-scores of patient TMA cases. (B) Mean LINE1 H-scores displayed in ascending order, to illustrate distribution of scores across cases.

Expression of LINE1 was found to vary between TMA cases (Figure 2, Figure 3). A distinction was seen between 3 patients with greater LINE1 expression, which were then defined as LINE1-high, and the other 10 cases (LINE1-low).

4.2.2 Immune infiltration

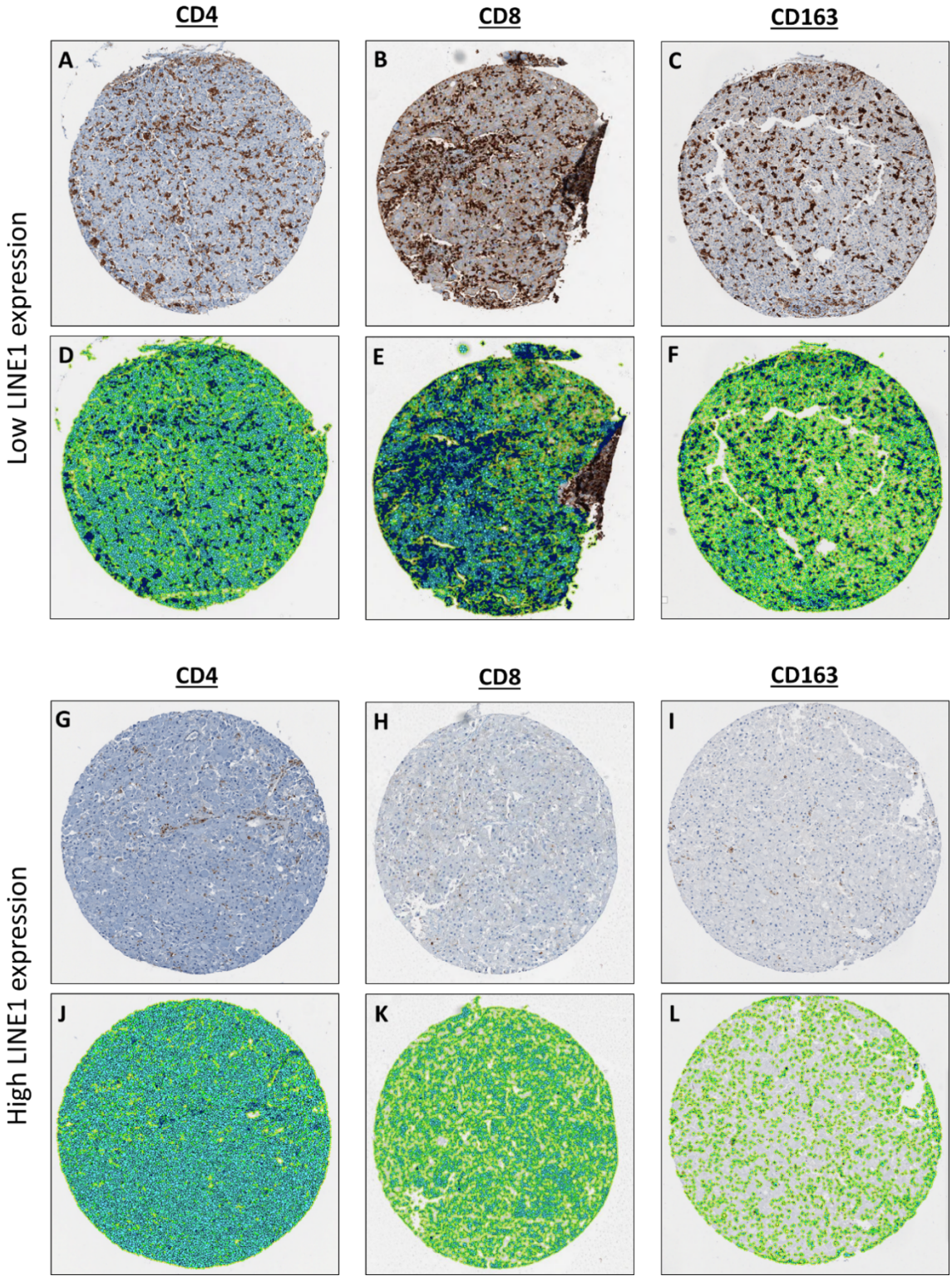

Figure 4: Immunohistochemistry images from Aperio ImageScope of TMAs from two different tumours, one expressing low levels of LINE1 (A-F), and one expressing high levels of LINE1 (G-L). TMAs were stained for CD4 (A, D, G, J), CD8 (B, E, H, K), and CD163 (C, F, I, L).

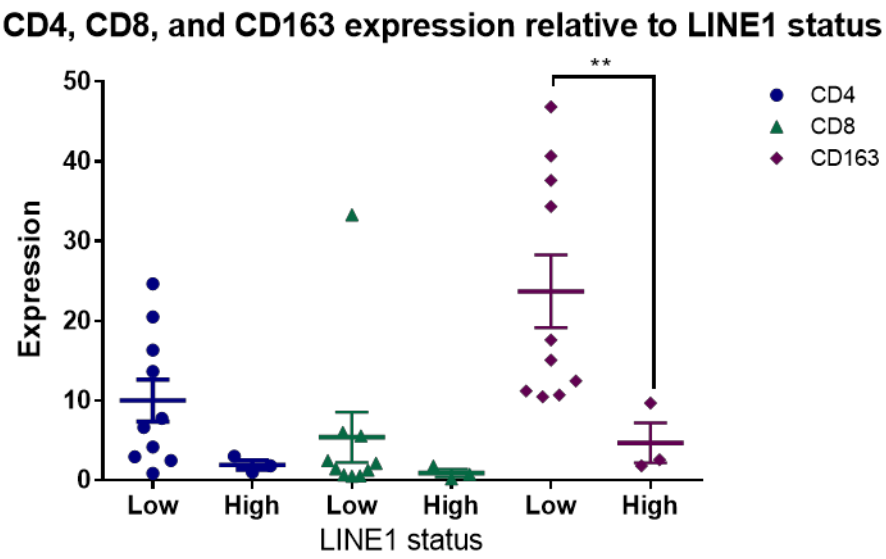

Figure 5: Expression of CD4 (A), CD8 (B), and CD163 (C) shown relative to tumour LINE1 expression status. Mann-Whitney U test: (A) P=0.1119, (B) P=0.2867, (C) P=0.0070.

Although expression of CD4, CD8, and CD163 classified by tumour LINE1 status all follow an expected trend, only CD163 expression was significantly lower in patients with high than low LINE1 status (P = 0.0070).

4.3 TGF-β

4.3.1 A significant association was found between LINE1 expression and TGF-β signalling status

To explore the association between LINE1 and TGF-β identified in exploratory studies by the group, firstly LINE1 expression was evaluated with regards to TGF-β signalling status[16].

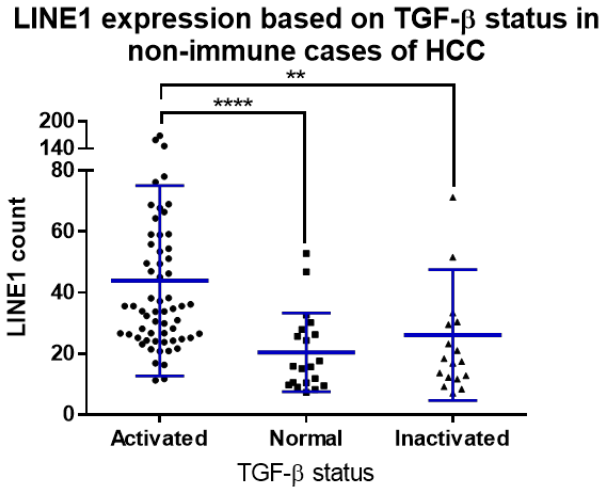

Figure 6: LINE1 expression and TGF-β status in non-immune cases of HCC. TGF-β status is shown as activated, normal, and inactivated, based on previous research by Chen et al. which created these classifications from TCGA RNAseq data. N=98. Kruskal-Wallis test P<0.0001.

LINE1 expression varied according to TGF-β status, with significantly greater LINE1 expression when TGF-β signalling was activated than when normal or inactivated (P<0.0001, P=0.0025).

4.3.2 Correlation analysis found an overall trend of positive correlation between the TGF-β signalling family and LINE1

12 of the 18 members of the TGF-β signalling superfamily had significant positive correlation with LINE1. Analysis was initially performed in all cases, then limited to non-immune cases to determine whether signalling was coming from cancer cells or immune infiltration.

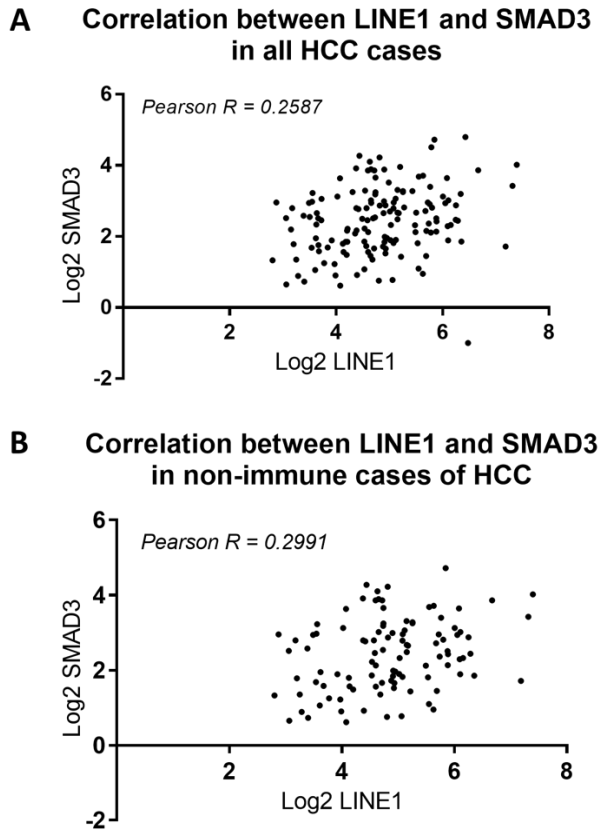

Figure 7: Correlation analysis between LINE1 and SMAD3 in (A) all HCC cases (R=0.2587, P=0.0016, n=147), and (B) only non-immune cases of HCC (R=0.2991, P=0.0021, n=103).

The correlation between LINE1 and SMAD3, a downstream component of the TGF-β signalling pathway, was significant (Figure 7). Significance was found when analysis was performed on all cases of HCC, and also when limited to non-immune cases.

4.3.3 Immunohistochemistry showed variations in pSMAD3 expression between tumours

Cell block staining confirmed a positive association between pSMAD3 and LINE1 expression: pSMAD3 expression was greater in the LINE1-high cell block (HUH7 WT) than LINE1-low (HUH7 LINE1-KD).

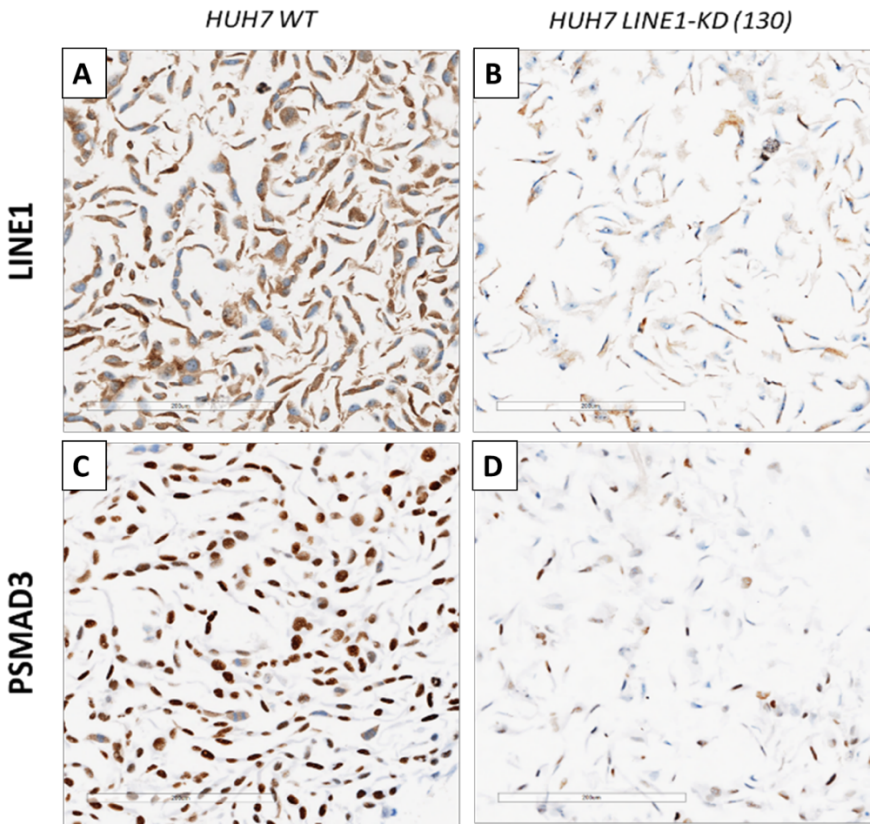

Figure 8: Staining of cell blocks for LINE1 (A, B) and pSMAD3 (C, D). Staining was performed on HUH7 WT cells (A, C), and HUH7 LINE1-KD cells (B, D). HUH7 WT cells are expected to have high LINE1, whereas LINE1-KD cells are expected to have low LINE1. LINE1 expression is predominantly cytoplasmic. pSMAD3 staining is predominantly nuclear.

Correlation analysis showed significant positive correlation between LINE1 and SMAD3 (Figure 7).

Immunohistochemistry analysis was performed to look for pSMAD3 protein expression as pSMAD3 is only present when activated, thus is more specific than SMAD3.

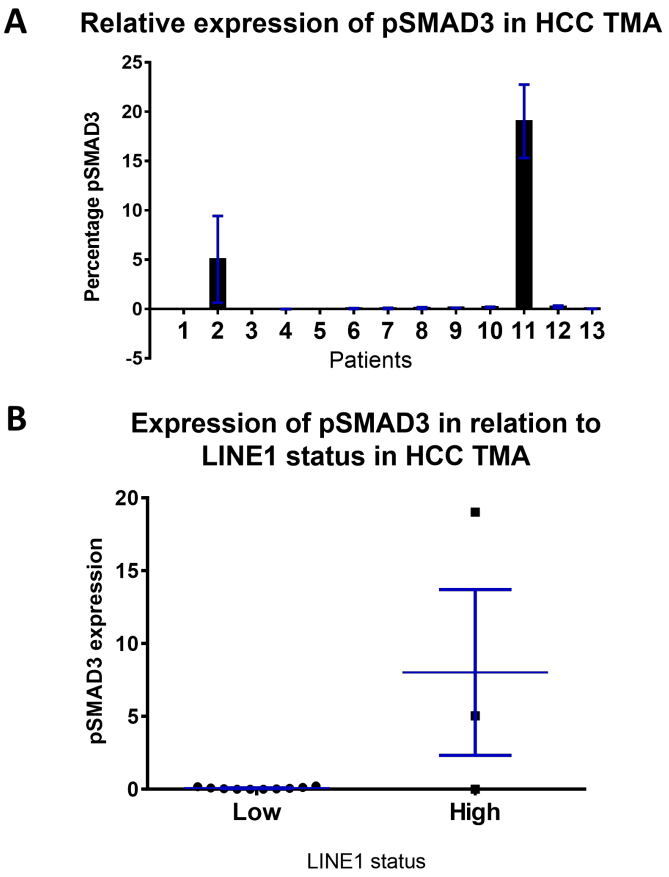

Figure 9: Immunohistochemistry staining of TMA for pSMAD3. (A) Relative expression of pSMAD3 in 13 HCC tumours. (B) Expression of pSMAD3 in relation to LINE1 status (high or low). N = 13 samples. Mann-Whitney U test P = 0.2937.

One-way ANOVA determined no statistically significant difference between pSMAD3 expression in samples with high and low LINE1 status, however the trend follows expectations.

5 DISCUSSION

5.1 The findings support an association between LINE1 and immune status

The findings support the hypothesis that LINE1 expression is greater in non-immune HCC, and add to previous literature demonstrating that LINE1 is an important element in HCC[12, 13]. RNAseq analysis showed LINE1 expression is greatest in the non-immune class, thus these findings have prognostic implication[10]. Immunohistochemistry supported these RNAseq findings.

The results do not explore whether LINE1 makes the immune microenvironment less immunogenic, or whether tumours with low immune activity are more prone to LINE1 insertion.

Limitations of immunohistochemistry include lack of tumour heterogeneity, small sample size, and the process of tumour resection can stimulate an acute injury response, which may cause increased presence of TILs. However, this response would not account for interpatient variations.

Differences in CD4, CD8, and CD163 expression were consistent with previous research demonstrating variations in immune environment in HCC[10]. Significant difference in CD163 expression based on LINE1 status was interesting, as CD163 is a marker of the exhaustive immune subclass, which RNAseq determined to have lowest LINE1 expression.

More understanding of the underlying immune changes and how they occur is required to determine how to exploit these responses and potentiate immunotherapy.

5.2 A novel positive association has been indicated between LINE1 and TGF-β in HCC

A TGF-β/LINE1 axis has previously only been shown in NSCLC[17]. This association may be key to understanding the relationship between LINE1 and the immune system in HCC. Overall, TGF-β superfamily transcript analysis was positive both when analysing all cases and when limited to non-immune cases, which confirms tumoural secretion was significant.

Although 6 members of the TGF-β superfamily had no significant correlation with LINE1 at transcript level, association at other levels of expression was not assessed. Protein and post-translational expression should be assessed in the future.

Whilst TMA analysis showed no statistically significant difference between expression of pSMAD3 in high and low LINE1 expressing tumours, all LINE1-low tumours had exceptionally low levels of pSMAD3 in comparison to 2 of 3 LINE1-high tumours. TMAs were created from tumours removed during surgery, and pSMAD3 will not remain phosphorylated in the hours it takes to resect the tumour, formalin fix, and process the tissue. Assessing pSMAD3 in biopsy tissue would help overcome this issue, as biopsy tissue is processed much more rapidly so pSMAD3 would be seen.

6 CONCLUSIONS

The primary aim of this project was to evaluate the relationship between LINE1 retrotransposon expression status and the immune microenvironment in HCC. The most significant finding to emerge from this study was an inverse association between LINE1 status and tumour immune activity. This is the first study to demonstrate this association in HCC, and these findings will be useful in developing understanding of why immune changes occur in HCC.

The second major finding was a relationship between LINE1 and TGF-β signalling in HCC. This association may help to explain why LINE1-associated changes in tumour immune microenvironment occur.

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