LINE-1 ORF1p Is an Early Diagnostic Marker of Cancer and Its Precursor Syndromes

PhD Dissertation

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LIST OF PUBLICATIONS RELATED TO THE SUBJECT OF THE THESIS

I. Karkas R, *__Abdullah KSA__, Kaizer L, Ürmös Á, Raya M, Tiszlavicz L, Pankotai T, Nagy I, Mátés L, Sükösd F. LINE-1 ORF1p is a Promising Biomarker in Cervical Intraepithelial Neoplasia Degree Assessment. Int J Gynecol Pathol. 2024 Jun 26. (Impact factor 3.32 (Q1)).

II. Imre et al. Prolonged activity of the transposase helper may raise safety concerns during DNA transposon-based gene therapy. Mol Ther Methods Clin Dev. 2023 Mar 14;29:145-159. (*Impact factor 5.84 (Q1)*)

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LIST OF OTHER PUBLICATIONS DURING PhD STUDY

Full Papers:

I. Kopasz et al. A versatile transposon-based technology to generate loss- and gain-of-function phenotypes in the mouse liver. BMC Biol. 2022 Apr 1;20(1):74. doi: 10.1186/s12915-022-01262-x. PMID: 35361222; PMCID: PMC8974095 (*Impact factor 7.43 (Q1)*)

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INTRODUCTION

Long Interspersed Nuclear Element-1 (LINE-1 or L1) retrotransposons are autonomous mobile genetic elements that cover approximately 17% of the human genome , stand for the most abundant class of transposable elements. These elements play a pivotal role in genome plasticity and evolution, contributing to genetic diversity and potentially adaptive changes. Two open reading frames (ORFs), which encode for L1ORF1p and L1ORF2p. ORF1, encodes for a ~40 kDa protein (L1ORF1p) with RNA-binding capacity that forms flexible homotrimers and displays an RNA-binding function. In age-related diseases, an increase in L1 activity has been observed in aged tissues and has been linked to cellular senescence. Numerous studies have demonstrated elevated levels of L1 expression in a range of cancer types, indicating that L1 activity may play a role in the initiation, progression and genomic instability associated with cancer. Cervical cancer is a significant international health problem, with a remarkably high incidence among women of reproductive age. It

progresses in the cervix, the lower part of the uterus that links to the vagina and is often preceeded by precancerous transformations, known as cervical intraepithelial neoplasia (CIN). CIN is categorized into three grades (CIN 1, 2, and 3) depending on the extent of abnormal cell growth. CIN I is a low-grade squamous lesion (LSIL) caused by low-risk HPV types that often go away on their own in more than three-quarters of cases. CIN II and CIN III are high-grade squamous lesions (HSILs) that have a higher risk to develop to a cervical cancer. The diagnosis of cervical cancer and/or CIN typically necessitates a combination of screening tests. The Papanicolaou (Pap) smear test remains a fundamental component of cervical cancer screening. The diagnosis of CIN is primarily depended on the histological assessment of cervical biopsy samples. Though, interobserver alterability and subjectivity in the explanation of histopathological characteristics of CIN lesions can impact diagnostic precision and reproducibility. Therefore, there is an extending need for objective and trustworthy biomarkers that can support traditional diagnostic approaches and improve the accuracy of CIN diagnosis. The most

commonly utilised immunohistochemical markers for CIN are Ki67 and p16. But there are gaps in utilising these markers for low-grade dysplasia where Wentzensen et al. observed that the specificity of p16/Ki-67 dual staining reduced considerably when employed for the identification of CIN1, which could result in overdiagnosis and unnecessary interventions. These uncertainties show up the necessity for caution when utilising Ki-67 and p16 IHC for CIN1 diagnosis. Furthermore, they emphasise the importance of investigating new reliable markers to classify CIN1 cases and avoid low-grade cervical lesions misdiagnosis.

Aims of the study

The objective of the present study is to investigate the expression of LINE-1 ORF1p in normal and malignant tissues, with a view to exploring its potential as a biomarker for cancer progression and response to therapy. The central concept of our research is to characterise the role of LINE1 retrotransposition in carcinogenesis by studying the expression pattern of LINE-1 ORF1p in various malignant tissues.

2. MATERIALS AND METHODS

Tissue specimens and ethics statement

The study was conducted on 590 samples derived from 21 distinct tumour types, in addition to CIN I (n=20), CIN II (n=46), CIN III (n=14), cervical cancer (n=32), non-dysplastic cervical tissue samples (n=31) and normal tissues from various organs (n=36). The control group included 31 cases of non-dysplastic cervical tissue taken from patients who submitted to total hysterectomy.

H&E and Immunohistochemistry (IHC) staining

TMA slides were incubated with three changes of xylene, followed by rehydration through two changes each of 100%, 95%, 70%, and 30% ethanol. Once the TMA slide was removed and rehydrated, the process was repeated. Subsequently, the sections were stained in Meyer's hematoxylin and eosin solution, followed by dehydration and mounting. IHC staining of the selected specimens was conducted on the Bond Max Autostainer with the Bond Polymer Detection System. anti-p16 antibody, anti-Ki67 and anti-LINE-1 ORF1p were used. The intensity of

staining across all tissue samples of were evaluated by an experienced pathologist using a semiquantitative approach.

Plasmid construction

To study the effects of P53 knockdown on LINE-1 retrotransposition in cell culture, we modified a construct we have already developed. In this the neomycin selective marker gene was cloned to the A side (HADHA) of the human hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex alpha and beta bidirectional promoter. The neomycin gene is disrupted with a short intron from which an artificial microRNA (amiR) is expressed to silence the TP53 gene. On the B side (HADHB) of the bidirectional promoter we cloned an ORFeus-type LINE1 reporter element. These transcriptional units were cloned between the two inverted terminal repeats (ITRs) of the Sleeping Beauty transposon.

Cell culture, transfection, fluorescence-activated cell sorting (FACS) analysis and paclitaxel treatment

The impact of TP53 expression on LINE1 retrotransposition was investigated in HepG2, HT-1080 (with normal TP53 function) and Saos-2 (with TP53 deletion) cell lines, which were transfected with 500 ng of the LINE-1 ORFeus reporter construct in combination with 50 ng of the hyperactive SB100 transposase helper plasmid, using FuGENE® HD transfection reagent (Promega) in accordance with the manufacturer's instructions. The rate of LINE-1 retrotransposition was measured with FACS. To test the effect on cancer therapy on LINE-1-ORF1p expression, cells were treated with 5 nM final concentration of paclitaxel in the culture medium.

3. RESULTS AND DISCUSSION

3.1 LINE-1 ORF1 PROTEIN EXPRESSION

L1 ORF1p is not present in normal somatic tissues but is expressed in malignant tissues

we employed IHC to survey LINE-1 ORF1p expression in mature somatic tissues from two male and two female autopsy cases on 36 different organs. The results demonstrated low levels of expression of LINE-1 ORF1p in striated muscles, including skeletal muscles, myocardium, and oesophageal striated muscle fibres. Majority of other normal mature somatic tissues exhibited an absence of expression of LINE-1 ORF1p. Then we test LINE-1 ORF1p expression in various malignant tumours, we found approximately 355 of the 590 cases (57%) exhibited a notable degree of intermediate and high ORF1p immunoreactivity. LINE-1 ORF1p expression was higher in skin basal cell carcinoma (100%), cervical cancer (83.87%), oesophageal cancer (70%), and non-small cell lung cancer (59%).

LINE-1 ORF1p expression correlates with cancer progression

We examined the expression pattern of LINE-1 ORF1p across different clinical stages and progression of cancer. The findings revealed that the LINE-1 ORF1p expression was elevated in high-grade tumours and advanced stages in comparison to low-grade and non-advanced tumours.

LINE-1 ORF1p expression shows intratumoral heterogeneity in some cancers We analysed LINE1 ORF1p expression by IHC on tissue microarray (TMA) cores in a wide range of tumour types. We found a significant degree of cellular variability was observed within the tumours, with some cells exhibiting high ORF1p expression in the peripheral regions while others showed low expression in the central region. Additionally, unicellular heterogeneity was identified, where individual cells exhibited considerable differences in ORF1p expression levels.

3.2 LINE-1 ORF1p AND TUMOUR SUPPRESSOR GENES

LINE-1 ORF1p expression is increased in TP53 immunoreactive malignant tissues To investigate the hypothesis that acquired mutations in certain tumour-suppressor genes may correlate with LINE-1 ORF1p expression, our cohort samples were also stained with anti-human P53 antibody by IHC. Our findings revealed that ORF1p expression occurred more frequently in the absence of functional p53 protein. The regression analysis was employed to compare the overall frequencies and percentages of ORF1p and TP53. A moderate significant positive correlation was observed between ORF1p and TP53 immunoreactivity (Spearman r = 0.46, p = 2.659e-30).

Endogenous TP53 silencing in vitro increases the rate of LINE-1 retrotransposition

To corroborate the findings of the tumour bank study, we developed an in vitro assay system to investigate the effect of endogenous TP53 gene silencing on L1 retrotransposition. We found a significant increase in

LINE1 retrotransposition in case of P53 knockdown compared to normal P53.

3.3 NEOADJUVANT TREATMENT INCREASES LINE1 EXPRESSION *Neoadjuvant treatment associated with increased ORF1p immunoreactivity*

Our results demonstrated a notable elevation in L1 ORF1p expression among tumour specimens derived from patients who underwent neoadjuvant treatment particularly cancers of the uterus, lung (SCLC), ovary, breast and pancreas, when compared to the untreated cohort. This observation suggests that antitumour therapy may potentially induce L1 expression. To further validate this relationship, HepG2 cells were transfected with a LINE-1 reporter construct and the SB100 transposase. Following the complete selection with neomycin, one group was treated with 5nM paclitaxel for 72 hours, while the other remained untreated. We found the paclitaxel-treated group exhibited 42.0% L1 retrotransposition, while the untreated group demonstrated 37.6%. These findings offer insight into the potential impact of cancer treatments such as paclitaxel on L1 elements activity.

3.4 LINE-1 ORF1p IS A GOOD BIOMARKER FOR CERVICAL CANCER.

While evaluating the LINE-1 ORF1p IHC staining results of tumour samples, a specific immunoreactivity pattern was observed in cervical cancers. Therefore, a more comprehensive investigation was conducted of additional cervical tissue samples. The expression of ORF1p, Ki67, and p16 was evaluated through immunohistochemical staining for entire samples.

LINE-1 ORF1p is expressed from early stage of cervical neoplasia.

We found that none of these markers are expressed in normal cervical tissue. However, as the tissue progresses to CIN I, a slight increase in marker expression becomes evident, particularly in the lower third of the epithelium for Ki67 and P16, while ORF1p exhibits more pronounced expression in the upper two-thirds. The transition to CIN II is characterised by a substantial increase in all markers, with expression typically

spanning the lower two-thirds of the epithelium. This trend intensifies in CIN III, where Ki67, P16, and ORF1p all demonstrate strong, transepithelial expression throughout the entire thickness of the epithelium. In malignant tissue, all three markers display extensive and intense staining across the disorganized tissue architecture. The ORF1p diverged from the typical pattern observed in CIN I, demonstrating a rising expression with higher grades of CIN towards cancer. In contrast, it was barely or not observed in normal cervical epithelium.

ORF1p staining is a reliable test to diagnose cervical cancer cases

To evaluate the accuracy of ORF1p IHC as a diagnostic test for neoplastic and dysplastic cervical cancer, we assessed the diagnostic test performance parameters. The calculated positive predictive value (PPV) and negative predictive value (NPV) of ORF1p staining which were 77% and 100%, respectively, when compared to non-dysplastic samples, indicating a high degree of accuracy in the detection of CIN I. The test result exhibits an exceptionally high level of sensitivity, with a narrow

confidence interval approaching 100%. This finding is consistent with the absence of false negatives. The specificity is also satisfactory at approximately 80%, although with a wider confidence interval, which reflects the presence of some false positives.

THE MOST IMPORTANT NEW RESULTS:

The most important new results of my PhD thesis are as follows:

1. ORF1p is typically absent or expressed at low levels in normal somatic tissues, except for some expression in striated muscles and the basal layer of the epidermis
2. ORF1p is often found in human cancers, with 57% of cases showing high levels of expression with high frequency in skin basal cell carcinoma, cervical cancer, oesophageal cancer, and non-small cell lung cancer.
3. The expression of ORF1p has been demonstrated to correlate with the tumor progression where it

was positively correlated with histological grad and pathological stage.

4. A significant increase in L1 ORF1p expression was observed in patients undergoing neoadjuvant treatment compared to the untreated group. This suggests that antitumour therapy may upregulate L1 expression.
5. There is considerable intratumoral heterogeneity in LINE-1 ORF1p expression, particularly in endometrial cancers.
6. The results demonstrated that cancers with overexpression of TP53, mutations in BRCA1/2 and HER2 exhibited significantly higher levels of ORF1p immunoreactivity.
7. ORF1p exhibited aberrant expression patterns from the CIN I stage onwards, preceding the established markers Ki67 and p16, with high sensitivity and specificity for detecting CIN I and invasive cervical cancer makes it a valuable diagnostic tool.