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Human Ligase 3 (LIG3) Impact on Primary Surgically Resected Epithelial Ovarian Cancer is Dependent on Its Cellular Sub-Localization - Clinical and Preclinical Evaluation

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Abstract

Background: DNA Damage Repair (DDR) proteins play crucial roles in tumour behavior. LIG3 is key cell DDR element. This study evaluates the impact of LIG3 subcellular localization in primary Ovarian Cancer (OC) patients who were treated by upfront surgery.

Methods: LIG3 nuclear and cytoplasmic expression was assessed pre-clinically by performing nuclear and cytoplasmic extraction on A2780 (Platinum sensitive) and A2780Cis (Platinum resistant) OC cell lines. Western blot was utilized to identify the representative protein bands. GraphPad Prism software was utilized to perform the analysis.

Results: After 48 h of incubation with cisplatin, A2780 cells showed a significant increase in LIG3 cytoplasmic fraction, (p-value = 0.0072) compared to the untreated cells. Similarly, in A2780Cis an increase in cytoplasmic fraction of LIG3 was observed but it did not reach a statistically significant value, (p-value = 0.2854). On the other hand, the nuclear isoform of LIG3 did not show any statistically significant change in both platinum sensitive and resistant cell lines, (p-value = 0.1014) and (p-value = 0.7510) respectively.

Conclusion: Human cytoplasmic LIG3 has crucial role in OC progression and can potentially predict patients' outcome and resistance to platinum chemotherapeutic agents. The data presented here reveal novel findings with vital clinical applications that could be used for future patients' treatment categorizations.

Keywords: DNA repair; LIG3; Nuclear; Cytoplasmic

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Introduction

The environmental factors often repeatedly cause damage to the DNA [1]. Carcinogenesis will be the outcome if such DNA damage is not efficiently repaired by the host DNA repair system. Thus, the DNA restorative ability plays a critical role in maintaining the stability of the human genome [1]. DNA ligases play an essential role in maintaining genomic integrity [2]. There are three human genes, which encode ATP-dependent DNA ligases: Ligase I (LIG1), Ligase III (LIG3) and Ligase IV (LIG4) [3].

LIG3 gene encodes three different DNA ligase polypeptides, Mitochondrial and Nuclear versions [4]. DNA LIG3 dependent repair of DNA breaks in the nucleus is critical for neuronal cell viability. Thus, mutations in LIG3 can result in inherited neurodegenerative syndromes [5]. Moreover, LIG3 deletion resulted in embryonic lethality in mice [6]. In cancerous tissue, an elevated steady-state level of DNA LIG3 was identified as biomarker of altered DNA DSB repair in Breast cancer, leukemia's and neuroblastoma [7-9]. Interestingly, LIG3 deficient cell lines were hypersensitive to DNA-alkylating agents [3].

Supplementary Table 1: Ovarian cancer cell lines used for initial testing, including growth medium, tumor/cell type and source of purchase.

Cell Line	Growth Media	Primary Tumour	Cell Type	Properties	Purchased
A2780	RPMI (Thermofisher Scientific) - 10% FBS (F4135, Merck, UK), 1% Penicillin-Streptomycin (P4333, Merck, UK).	Carcinoma	Ovarian Cancer	Cisplatin Sensitive	Sigma Aldrich (Gillingha, UK)
A2780cis	RPMI (Thermofisher Scientific) - 10% FBS (F4135, Merck, UK), 1% Penicillin-Streptomycin (P4333, Merck, UK).	Carcinoma	Ovarian Cancer	Cisplatin Resistant	Sigma Aldrich (Gillingha, UK)

We hypothesized that Human LIG3 is an important signaling transduction molecule that has diverse function in different cancerous organs. Previously we have shown that LIG3 clinical influence on primary Ovarian Cancer (OC) is dependent on its cellular sub-localization [10]. The aim of this study is to identify if this difference is still evident in preclinically.

Methods

Compounds and antibodies

LIG3 antibody was purchased from Sigma-Aldrich (HPA006723). Cisplatin solution (1 mg/ml) was obtained from the Department of Pharmacy, Nottingham University Hospitals, Nottingham, UK.

Sub culturing cell lines

A2780, platinum sensitive (human ovarian carcinoma, isolated from primary tumors of untreated patient) and A2780Cis, Cisplatin-resistant (cell line developed by continuous exposure of A2780 to cisplatin doses) were utilized for the experiments. Both cell lines were obtained from the American Type Culture Collection; ATCC, Manassas, USA. Both were grown using 1% penicillin/streptomycin +10% Fetal Bovine Serum (FBS) RPMI (1640) at 37°C with 5% CO_2/95% air, Supplementary Table 1. Authentication of cell lines were done by ATCC Company using Promega's PowerPlex® 17 Short Tandem Repeat (STR) systems to ascertain there was no cross-contamination or misidentification of cell lines. Mycoplasma test was routinely performed almost every six months using MycoProbe Mycoplasma Detection Kit (R&D systems; Abingdon, UK). Cell lines were used up to 16 passages.

To trypsinise cells, each cell line was separately handled under sterile conditions with its own reagents. First, cell media was removed by pipetting, and then cells washed by PBS (without Mg2+ and Ca2+). Subsequently, 0.5 mg/ml trypsin- EDTA (Sigma Aldrich, UK) was added to disrupt cell monolayers. Then, all cells were incubated for 3 min to 5 min at 37°C; medium was then, added to the flask 4:1 in order to deactivate trypsin. Centrifuge at 1,000 rpm for 5 min for cells was the next step. Followed by, cell pellet was re-suspended in fresh media, which was then mixed well and placed into a new flask.

Western blotting

Cells were lysed in RIPA buffer (Sigma Aldrich) complemented with protease inhibitor (Sigma) and phosphatase inhibitor cocktail 1 and 2 (Sigma). Protein quantification was performed using BCA colorimetric kit (Thermofisher, UK). Samples were run on SDS-PAGE gel (4% to 12%) bis-tris. Membranes were incubated with anti LIG3, anti-GADPH and anti-YY1 for 1 h room temperature. Membranes were then labeled with infrared dye labeled secondary antibodies (Li-cor) [IRDye 800CW Donkey Anti-Rabbit IgG (H+L) and IRDye 680CW Donkey Anti-Mouse IgG (H+L)] for 1 h at room temperature. Protein detection and quantification was determined by scanning the membranes on Licor-Odyssey's Scanner (Licor, Biosciences) at the predefined intensity fluorescence. For bands quantifications, the Image Studio Lite software (ver 3.1) (Li-Core, USA) was used. Western blot was performed in three independent settings. Differences between samples were calculated using student t-test. Graphs and statistical analysis were performed using GraphPad Prism software version 7.A p value of less than 0.05 was identified as statistically significant.

Nuclear and cytoplasmic extraction

Cells from both A2780 and A2780Cis cell lines were seeded in T25 flasks overnight. Cisplatin of 5 µM was then added to the cells and incubated for 24 h and 48 h. Then, cells were collected by trypsinization, centrifuged at 1,000 rpm for 5 min at room temperature. The cells were then transferred to 1.5 ml Eppendorf tubes. Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Kit was utilized for separation of nuclear and cytoplasmic fractions and according to the manufacturer's instructions (78833, Thermo Fisher; UK). The cells were washed with cold PBS leaving the cell pellet as dry as possible, after the PBS was carefully discarded. The cells were then incubated with 200 µl of Cytoplasmic Extraction Reagent (CER) I and vigorously vortex the tubes for 15 sec, then incubated on ice for 10 min. 11 µl of cold-CER II was added to the tubes and the cells were vortexed vigorously for 5 sec and incubated on ice for 1 min. After that, the cells were vortexed for another 5 sec and centrifuged at maximum speed in a microcentrifuge for 5 min. The supernatants were immediately transferred to clean Eppendorf tubes and placed on ice. The nuclei in the cell pallet were incubated with 100 µl cold-Nuclear Extraction Reagent (NER), vortexed for 15 sec and placed on ice. The cell pallet vortexed for 15 sec every 10 min for a total of 40 min. The pellets were then centrifuged at a maximum speed in a microcentrifuge for 10 min. The nuclear extracts were immediately transferred to clean tubes and placed on ice. Extracts were quantified using BCA protein quantification kit, and protein levels were checked by utilizing western blot technique. Nuclear and cytoplasmic extracts were blotted against YY1 as a nuclear marker and GAPDH as a cytoplasmic abundant marker in order to verify the purity of the extractions. Proteins in the nuclear extractions were normalized to YY1 levels. Protein levels in cytoplasmic extracts were normalized to GAPDH. This study was carried out in accordance with the declaration of The Helsinki and ethical approval, which was obtained from the Nottingham Research Ethics Committee (REC Approval Number 06/Q240/153).

Results

LIG3 levels were evaluated in nuclear and cytoplasmic extracts from platinum sensitive and resistant OC cell lines, A2780 and A2780Cis respectively before and after 48 h of cisplatin treatment. Although after 24 h we haven't noted a significant difference in LIG3 level, at 48 h, in platinum sensitive cell lines, it revealed a significant increase in the cytoplasmic fraction of LIG3, (p-value = 0.0072) compared with the untreated cells. Similarly, an increase in nuclear fraction of LIG3 was observed but it did not reach a statistically significant value, (p-value = 0.1014) (Figure 1A, 1B). On the other hand, in A2780Cis cells both cytoplasmic and nuclear levels of LIG3 showed no significant changes after cisplatin treatment, (p-value = 0.2854) and (p-value = 0.7510) respectively (Figure 2A, 2B).

Discussion

Normal cellular metabolic processes in mammals can result in

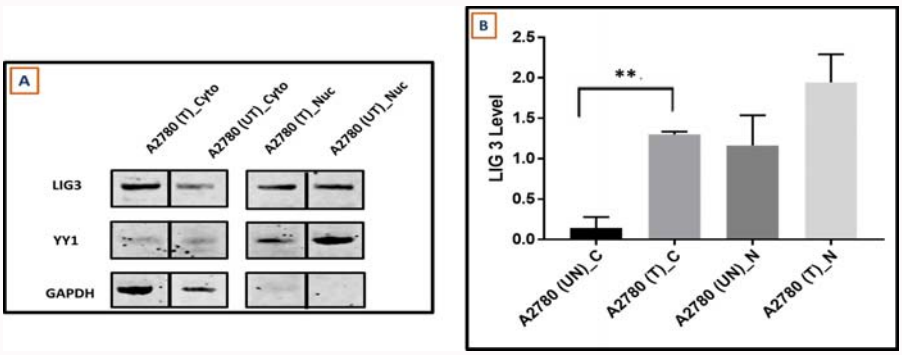

Figure 1: A) Representative Immunoblots of nuclear and cytoplasmic levels of LIG3 in A2780 ovarian cancer cells, B) Quantification of cytoplasmic and nuclear levels of LIG3 after adjustment with the loading control. GAPDH was used as a loading control for the cytoplasmic fractions and YY1 as a loading control to the nuclear fractions.

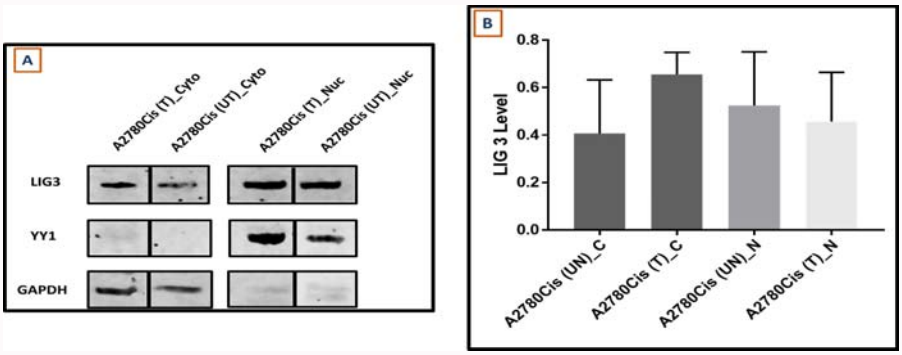

Figure 2: A) Representative Immunoblots of nuclear and cytoplasmic levels of LIG3 in A2780Cis ovarian cancer cells, B) Quantification of cytoplasmic and nuclear levels of LIG3 after adjustment with the loading control. GAPDH was used as a loading control for the cytoplasmic fractions and YY1 as a loading control to the nuclear fractions.

byproducts with potential cellular toxicity [11]. These can result in the production DNA damage which [12,13]; if left unrepaired, can cause cell death. However, if this DNA damage is miss-repaired, it can lead to genomic instability and in turn facilitate cancer development or progression [14,15]. The three human ligases, LIG1, LIG3 and LIG4 have critical roles in maintaining the genomic integrity [16]. They are involved in DNA replication; genetic recombination and DNA repair [3,17,18]. LIG3 is a biomarker of altered DNA Damage Repair (DDR). Compared with the other human ligases, LIG3 has the most robust intermolecular DNA joining activity [19]. Despite the major role of DNA LIG3 in rapidly proliferating cells, we are the first to report that LIG3 overexpression influence on primary OC is different as per its cellular sub-localization. We provided evidence that LIG3 cytoplasmic protein expression has the main clinical impact on primary OC patients.

Interestingly, high levels of LIG3 protein have been identified in different cancerous tissues, when compared with non-tumour specimens [7,8]. LIG3 gene encodes three distinct DNA ligase polypeptides: Nuclear LIG3α, mitochondrial LIG3α and a germ cell-specific LIG3β [20]. Although elevated level DNA LIG3β has been measured in testes, its mRNA has not been detected in the ovaries [21].

In our previous study, we have identified LIG3 as one of the differentially expressed proteins that showed high association with the cases' clinicopathological characteristics, platinum sensitivity and patients' prognosis [10]. Our results indicated a significant difference in the prognostic impact between the cytoplasmic and nuclear isoforms LIG3 protein in invasive OC patients. The cytoplasmic LIG3 expression showed pivotal impact on OC patients. Its high expression was inversely correlated with reduced tumour stage, lower tumour grade, less residual tumor following surgery, improved platinum sensitivity and outcome. Importantly, in multivariate analysis, cytoplasmic expression of LIG3 was an independent poor prognostic factor [10]. On the other hand, the nuclear LIG3 positive expression did not show a reliable prognostic relevance not just in univariate but also in multivariate analysis [10]. Importantly, in this study when we added cisplatin to OC cell lines, we observed a significant increase in the cytoplasmic fraction of LIG3 in platinum sensitive cell line, when compared to its nuclear samples. Similarly, in A2780Cis, cytoplasmic LIG3 showed increase in its levels, but it did not reach the statistical significance. This providing further preclinical evidence to the importance of cytoplasmic LIG3 in modulating platinum sensitivity in OC patients.

Interest in the underlying mechanisms regarding the role LIG3 cytoplasmic and nuclear subcellular localization in cancer progression is ongoing. A study on patients with Alzheimer's disease concluded that LIG3 deficiency in mitochondria can result in mitochondrial malfunction [22,23]. Moreover, Caldecott et al. [12] stated that the nuclear localization of LIG3 is dependent upon complex formation with XRCC1, a partner protein that has a nuclear localization signal [24]. Recently, it was reported that four isoforms of LIG3, which are produced by alternative splicing and alternative initiation, were identified. They were recognized in both the nucleus and cytoplasm mitochondria of the cells. Its mitochondrial isoform functions in

mitochondrial base-excision DNA repair. On the other hand, its nuclear counterpart interacts with the nuclear XRCC1 protein [25].

From these above studies, and from our results, we propose that the molecular regulation of LIG3 in OC is differentiated according to the subcellular localization. We speculate that the cytoplasmic staining of LIG3 observed in tumors could represent mitochondrial form of LIG3α. Altered mitochondrial DNA repair and replication capacity may influence response to platinum therapy, and increased LIG3α in the mitochondria could contribute to platinum resistance. However, further detailed mechanistic studies are required to confirm this hypothesis.

To conclude, DNA ligation is the last step in the process of DNA repair. It is a key event in finalizing the DNA repair cascade. Cytoplasmic LIG3 were strongly associated with unfavorable outcomes and platinum resistance, unlike its nuclear isoform. Our study provided novel findings and useful prognostic indications. However, it appeared that the underlying mechanisms of OC progression are contextualized with myriad biophysical and biochemical factors. More work is needed to elucidate the roles and relative contributions of the DNA LIG3 cellular sub-localization on ovarian tumour patients' prognosis and survival.

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