High inner centromere protein (INCENP) expression correlates with aggressive features and predicts poor prognosis in patients with invasive breast cancer.

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ABSTRACT

Introduction: Inner centromere protein (*INCENP*) is a member of the chromosomal passenger complex (CPC) and plays a key role in mitosis and cell proliferation. This study aims to evaluate the clinical and prognostic significance of *INCENP* in invasive breast cancer (BC).

Methods: INCENP protein expression was evaluated on a tissue microarray of a large BC cohort (n=1295) using immunohistochemistry. At the mRNA level, *INCENP* expression was assessed using the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) (n=1980) and Cancer Genome Atlas (TCGA) BC cohorts (n=854). The correlations between INCENP expression, clinicopathological parameters and patient outcome were investigated.

Results: INCENP protein expression was detected in the nucleus and cytoplasm of the tumour cells. Its expression was significantly associated with features characteristic of aggressive BC behaviour including high tumour grade, larger tumour size and high Nottingham Prognostic Index scores. High INCENP nuclear expression was a predictor of shorter BC-specific survival (BCSS) in the whole cohort, as well as in the luminal subtype ($p<0.001$). High INCENP nuclear expression was predictive of poor prognosis in BC patients who received hormone treatment or chemotherapy.

Conclusion: High INCENP expression is a poor prognostic biomarker in BC with potential therapeutic benefits.

INTRODUCTION

Cancer cells are characterised by uncontrolled cell proliferation, and cell cycle regulators are considered potential targets for cancer therapy [1]. Identifying mitotic regulatory proteins and key drivers of the cell cycle, specifically within the mitotic phase can be considered a powerful way to discover candidate genes that play effective roles in cell proliferation [2].

During mitosis, the chromosomal passenger complex (CPC) is a central regulator of chromosomal orientation, separation, and cytokinesis, and is required for genomic stability [3]. CPC can be regarded as a complex similar to cyclin/cyclin-dependent kinase (CDK) [4, 5]. INCENP (Inner Centromere Protein) is one component of the CPC that includes, Aurora kinase B, Survivin, and Borealin [6], and it binds directly to microtubules and is important for CPC localisation and function in mitosis [7]. It has two crucial roles in the CPC: first, it acts as a scaffold regulating CPC localisation and activity and organising complex assembly by interacting with the other three components; second, it interacts with Aurora kinase B, to activate the complex catalytic subunit [8].

Genome-wide association studies (GWAS) identified several single nucleotide polymorphisms (SNPs) in INCENP which contribute to the susceptibility of breast, ovarian, and prostate cancer [9]. INCENP is overexpressed in colorectal cancer [10], neuroblastoma cell lines [11], high-grade non-Hodgkin B-cell lymphomas and non-small-cell lung cancer and acts as a biomarker for poor prognosis [12, 13]. However, the role of INCENP in invasive breast cancer (BC), which is the most commonly diagnosed cancer worldwide [14] is still unclear. In this study, we aim to investigate *INCENP* expression in BC and investigate its relationship with clinicopathological features, and

outcomes at the protein and mRNA levels utilising large well characterised cohorts of BC.

MATERIALS AND METHODS

Principle of INCENP selection

As proliferation plays a major role in BC behaviour and prognostication, we aimed to identify genes associated with the proliferative activity of BC. A bioinformatic approach was used for the selection of key genes associated with high mitotic scores as a reliable measure of BC proliferative activity. Images of The Cancer Genome Atlas (TCGA) BC cases (n=1053) were utilised where mitotic figures were counted in full face invasive BC sections stained with haematoxylin and eosin (H&E) using digital whole slide images (WSI) of TCGA BC cohort. The TCGA data were analysed using the R (limma) package (http://bioconductor.org/packages/release/bioc/htm l/limma.html) and R language (R version 3.4.4; http://r-project.org/) was used to identify differentially expressed genes (DEGs) between high and low mitotic score cases. Data pre-processing including background correction, data normalisation, combining normal and tumour group data, ID transform gene symbol, and probe supplemental missing value was performed. Only genes with an adjusted $p < 0.05$ and log2FC >2 were selected as DEGs (where FC = fold change). Genes involved in mitotic cell division were identified. *INCENP* was the top significantly upregulated differentially expressed gene associated with a high mitotic score.

Immunohistochemistry study cohort

This study was conducted on a series of 1600 primary invasive BC cases diagnosed and treated between 1990 to 1998 at the Nottingham City Hospital, Nottingham, UK. Clinical information and tumour characteristics including patient's age at diagnosis, histological tumour type, grade, tumour size, lymph node stage, Nottingham Prognostic

Index (NPI), and lymphovascular invasion (LVI), were available [15]. Outcome data including BC-specific survival (BCSS), defined as the time (in months) from six months after the date of primary surgical treatment to the time of death due to BC, and distant metastasis-free survival (DMFS) defined as the time (in months) from six months after surgery until the first event of distant metastasis, were collected and calculated. Patients in this cohort were treated uniformly based on tumour features, NPI and hormone receptor status, according to the hospital protocol. Endocrine therapy was offered to post-menopausal women whose tumour was ER-positive (ER+) with moderate or poor NPI scores (> 3.4), while no adjuvant therapy was an option for patients with 'good' NPI scores (≤ 3.4). Premenopausal patients with moderate and poor NPI scores were subject to chemotherapy. The classical treatment of cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) was used as a therapy for ER negative patients who were clinically fit to receive chemotherapy. None of the patients in the current study cohort received neoadjuvant therapy.

Data for ER, PR, HER2 and Ki67 were available as previously published [15]. ER and PR positivity were defined as positive nuclear staining in ≥ 1% of the invasive tumour cells [16]. The proliferation index was evaluated using Ki-67 antibody immunohistochemical (IHC) staining and defined as high when ≥14% of tumour cells showed nuclear positivity [17] Immunoreactivity of HER2 was assessed using HercepTest guidelines. HER2 positivity was defined as strong positive complete membranous staining in ≥ 10% of the invasive tumour cells (score 3+). HER2 gene amplification status was assessed in borderline cases (IHC score 2+) using chromogenic in situ hybridisation (CISH), using the HER2 CISH pharmDx kit (Dako), as previously described [17, 18].

Cases were classified according to the molecular classification of BC: (i) Luminal A (ER and/or PR positive, HER2 negative and Ki67 <14%); (ii) Luminal B/HER2- (ER and/or PR positive, HER2 negative and Ki67 ≥14%); or Luminal B/HER2+ (ER and/or PR positive, HER2 positive); iii) HER2 enriched (non-luminal) (ER and PR negative and HER2 positive); and iv) Triple Negative breast cancer (TNBC) (ER, PR and HER2 negative).

Tissue microarrays and immunohistochemistry

Tissue microarrays were prepared from representative lesions of BC tissue as previously described [19]. In addition, a set of whole tissue sections from 10 cases containing invasive tumours were assessed to evaluate heterogeneity and the pattern of INCENP expression in malignant breast lesions, adjacent stroma, and normal tissue. Primary antibody specificity for rabbit monoclonal antibody INCENP, (Invitrogen, MA5-17100) was validated by Western blotting using cell lysates of MCF7, MDA-MB-231 and HELA human cell lines obtained from American Type Culture Collection, Rockville, MD, USA. INCENP antibody was used at a dilution of 1:500 which showed a single specific band at the predicted size of 105 kDa.

Expression of INCENP protein was assessed by IHC using the Novocastra Novolink polymer detection system (Code: RE7280-K, Leica, Newcastle, UK), where 4 □m tissue microarray and full-face sections were stained with the INCENP antibody (1:250) incubated for 60 minutes at room temperature. Antigen retrieval was performed in citrate buffer pH 6.0 using a microwave (Whirlpool JT359 Jet Chef 1000 W) for 20 min. 3,30-Diaminobenzidine tetrahydrochloride (Novolink DAB substrate buffer) was used as a chromogenic substance. Sections were counterstained with haematoxylin. Positive staining controls (human tonsil) were included while negative control was achieved by the omission of the antibody and by the application IgG of the same species following

the same staining protocol (Dako, polyclonal antirabbit immunoglobulins, REF: P0447, LOT:41236467, 1:1000) (Supplementary Figure 1).

Assessment of INCENP expression

The semi-quantitative H-score [20], considering both the intensity of staining and the percentage of stained tumour cells, of nuclear and cytoplasmic INCENP immunoreactivity was estimated. Cores containing <15% of tumour epithelial cells were excluded from the assessment. All cases were scored blinded to clinicopathological and outcome data. For dichotomisation of protein expression, cut-off points were defined according to the calculated results from X-tile bioinformatics software (Yale University, version 3.6.1) [21] with corrected *p*-value and relative risk against BCSS. High INCENP nuclear and cytoplasmic expression was considered when H-score was >100 in both.

Evaluation of INCENP mRNA expression

To confirm the prognostic significance of *INCENP* in BC, *INCENP* normalised mRNA expression was evaluated as a potential prognostic marker using the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) dataset that comprises 1980 tumours of invasive BC with comprehensive molecular characterisation and was used to evaluate *INCENP* gene copy number (CN) aberrations and gene expression [22].

The Illumina Human HT-12 v3 platforms (Illumina, Inc., San Diego, USA) were used in the METABRIC cohort to analyse/evaluate mRNA extracted from primary tumour samples. In TCGA (n = 854) [23]. RNASeqV2 data and clinicopathological information provided by the cBioPortal website were used [24]. Cut-off points used for dichotomising the INCENP expression in MEATBRIC and TCG cohorts were 6.4 and 277.3 respectively as determined using BCSS utilising X-tile software (Yale University, version 3.6.1).

For further validation of the prognostic significance of INCENP in BC, online external analytical modules were used, including the Breast Cancer Gene Expression Miner online dataset v4.3 (http://bcgenex.ico.unicancer.fr/BC), (n = 6291) [25], their dataset included DNA microarray data from METABRIC and Affymetrix and RNA-sequencing transcriptomic data from TCGA and Scan B. The Kaplan–Meier plotter (n = 1025) [26] was also used, and the sources for its database included Gene Expression Omnibus(GEO), European Genome-phenome Archive (EGA), and TCGA.

The clinicopathological parameters for the METABRIC and Nottingham series are summarised in (Supplementary Table 1). STRING database https://string-db.org/ was used to investigate other genes interacting with *INCENP.*

Statistical analysis

Statistical analyses were performed using SPSS v26 (Chicago, IL, USA) for Windows. Student's t-test and analysis of variance (ANOVA) were used to correlate between *INCENP* mRNA level as a continuous variable and other clinicopathological parameters in METABRIC and TCGA data. Association with *INCENP* mRNA expression and breast cancer-specific survival was performed after dichotomisation of expression into high and low groups based on the cut-off point obtained from X-tile software.

The correlation between *INCENP* mRNA expression and mRNA of other genes involved in cell proliferation was performed using the Pearson's correlation coefficient for continuous data. Association between INCENP expression and clinicopathological parameters in invasive BC was performed using Chi-square for categorised data, and Mann-Whitney and Kruskal-Wallis tests for continuous variables. The Spearman correlation test was used to compare the expression of INCENP between nuclear and cytoplasmic expression. Survival rates were determined using the Kaplan–Meier method and compared by the log-rank test. Multivariate analysis using the Cox

regression model determined the influence of INCENP expression, when adjusted to other variables, for BCSS and DMFS. All tests were 2-tailed and a *p*-value of less than 0.05 was considered statistically significant.

This study followed the criteria for the reporting recommendations for tumour marker prognostic studies (REMARK) **(**Supplementary Table 2) [27].

RESULTS

Frequency and localisation pattern of INCENP protein expression

Assessment of the whole tissue sections revealed nuclear expression of INCENP in invasive BC cells (Figure 1A) with occasional cytoplasmic staining (Figure 1B) with homogenous distribution patterns confirming the validity of using tissue microarrays to assess its expression. Epithelial cells in the adjacent normal breast terminal duct-lobular units showed negative or very weak cytoplasmic INCENP staining (Figure 1B and C). INCENP expression was detected in mitotic cells including normal and atypical mitoses (Figure 2).

After the exclusion of uninformative cases on the TMA i.e., lost, folded or cores containing scanty tumour cells <15% a total of 1295 were included in the analysis. Both nuclear and cytoplasmic INCENP expression showed a unimodal distribution with a median H score of 100 (range 0-300). Strong concordance was demonstrated when 20% of the cases were re-scored after 3 months wash-out period (ICC = 0.89, $p<0.001$ for nuclear expression and ICC=0.96, $p<0.001$ for cytoplasmic expression).

High nuclear INCENP expression was observed in 32.5%; while high cytoplasmic expression was seen in 16.3% of BCs (Figure 3).

A statistically significant correlation between nuclear and cytoplasmic INCENP expression was observed (r =0.49, $p=0.001$).

Association of INCENP protein expression with clinicopathological parameters

High INCENP nuclear expression showed significant association with features characteristic of aggressive behaviour including larger tumour size (p=0.001), higher tumour grade, higher mitotic scores, nuclear pleomorphism, less tubule formation, poor NPI, and high Ki67 labelling index (>14%), (p<0.001). High cytoplasmic expression was also significantly associated with higher grade, higher mitotic score, higher pleomorphism score (p<0.001), and less tubular development (p=0.014), In addition, high nuclear and cytoplasmic expression was significantly associated with invasive breast carcinoma of no special type (NST carcinoma) (Table1).

Association of INCENP protein expression and patient outcome

In univariate analysis, BC patients with high nuclear INCENP expression had a significantly poor outcome in terms of shorter BCSS (HR 1.64, 95%CI 1.27-2.10; p<0.001) and shorter DMFS (HR 1.57, 95%CI 1.24-1.99; p <0.001), respectively. When cases were classified according to the intrinsic molecular subtypes, high *INCENP* nuclear expression was associated with shorter survival in luminal A (BCSS: HR 1.98, 95%CI 1.34- 2.94; p<0.001 and DMFS: HR1.75, 95%CI 1.21-2.51; p=0.002) and luminal B (BCSS: HR 1.60, 95%CI 1.00-2.56; p=0.045 and DMFS: HR 1.58, 95%CI 1.04- 2.42; p=0.03); but not in TNBC or HER2 enriched classes (Figure 4).

In the multivariate Cox regression model in the whole cohort including other prognostic covariates (tumour grade, nodal stage, mitosis score and Ki67 score), nuclear INCENP was an independent predictor of shorter BCSS (HR 1.9, 95% CI 1.28-2.87, p=0.002) as well, as shorter DMFS (HR 1.83, 95% CI 1.24-2.69, p=0.003) (Table 2).

When the cohort was stratified based on the adjuvant therapy, high INCENP nuclear expression showed associated with shorter BCSS in patients who were given hormone therapy (HR1.68, 95%CI 1.21-2.32; p=0.002), chemotherapy (HR 2.44, 95%CI 1.22-4.88; p=0.009). Similarly, high INCENP was associated with shorter DMFS in patients

receiving hormone treatment (HR 1.5, 95%CI 1.1-2.04; p=0.01), as well as chemotherapy (HR 2.9, 95%CI 1.5-5.9; p=0.002) (Figure 5).

INCENP mRNA expression

A significant association was observed between high *INCENP* mRNA expression and *INCENP* gene CN gain (p<0.001) (Supplementary Figure 2). High *INCENP* mRNA expression (log2 intensity>6.4) was observed in 523/1969 (26.4%) of the METABRIC cases. High *INCENP* mRNA level was significantly associated with older age patients (p=0.008), post-menopausal status, larger tumour size, high tumour grade, poor NPI, invasive ductal carcinoma (NST), TNBC, and *TP53* gene mutation (all p<0.001), and high nodal stage (p=0.04). Analysis of the TCGA BC dataset showed similar significant results, in addition to the association with mitotic score (p<0.001) (Supplementary Table 3) and (Supplementary Figure 3).

The METABRIC and TCGA cohorts were used to examine the association between *INCENP,* and other genes involved in cell proliferation, such as *Ki67*, as well as cell cycle genes, such as *BUB1, CENPE, PLK1, CDCA8, CDC20, CDK1, KIF23, KIF20A, AURKA, and AURKB* at the mRNA expression level. As shown in (Supplementary Table 4)., there was a statistically significant association (p<0.001) between high expression of INCENP and genes involved in the cell cycle.

Survival analyses of the METABRIC cohort showed that high *INCENP* mRNA expression is associated with poor outcomes in terms of shorter BCSS (HR 2.06, 95%CI 1.77-2.54; p<0.001). According to the molecular subtypes, high *INCENP* mRNA expression was predictive of shorter BCSS in Luminal (HR 1.81, 95%CI 1.40-2.34; p<0.001) and TNBC (HR 1.77, 95%CI 1.16-2.75; p=0.008) but not in the HER2 enriched class (Supplementary Figure 4). Similarly, in the TCGA cohort, there was an association between high *INCENP* mRNA expression and poor patient outcome in all cases (HR

2.43, 95%CI 1.03-5.71, *p*=0.03) and in TNBC (HR 3.22, 95%CI 0.99-10.48, *p*=0.04) (Supplementary Figure 5).

The association between *INCENP* mRNA and aggressive features of the tumour were also validated and confirmed in the Breast Cancer Gene Expression Miner v4.3 database and the Kaplan–Meier plotter (Supplementary Figure 6).

DISCUSSION

The exact and timely coordination of chromosomal, cytoskeletal, and membrane trafficking events is essential for successful cell division. *INCENP* as a component of the CPC is one of the "chief regulators" of cell division. To the best of our knowledge, this is the first study to investigate the prognostic significance of *INCENP* in BC. Using IHC, we investigated *INCENP* expression and subcellular localisation in BC and discovered that, when expressed, INCENP was evident in the nucleus of the tumour cells, with occasional cytoplasmic expression. In a study by Barbanis et al, [12] INCENP was located in the nuclei of neoplastic lymphocytes as well as proliferating lymphoid cells, and this immunopositivity was found in all phases of mitosis as well as all atypical mitotic figures. Our results also revealed a positive correlation between INCENP nuclear and cytoplasmic expression in BC cells. It was reported that in the early stages of mitosis INCENP initially localise to the nuclei where they are tightly bound to the chromosomes and are concentrated at centromeres during metaphase, as they stimulate cell proliferation [5, 10, 28], then, at the metaphase/anaphase transition, they rapidly dissociate from the chromosomes and attach to the cytoplasmic microtubules of the central spindle [29]. During anaphase, a portion of INCENP translocate to the cleavage furrow and becomes involved in stabilising them [30] making it one of the earliest known markers for furrow assembly [31].

In this study we demonstrated that INCENP protein expression in BC is associated with clinicopathological parameters characteristic of poor prognosis including high tumour grade, high mitotic score and with shorter patients' survival, supporting its importance in BC progression. Our findings showed that high *INCENP* expression was also significantly associated with proliferation as assessed by the Ki67 labelling index. INCENP nuclear expression was an independent prognostic marker and significantly associated with shorter survival in the whole cohort, as well as, in the luminal tumours, which may have potential clinical relevance in improving survival rate prediction in luminal subtypes. Regarding adjuvant therapy, our results indicated that BC with high *INCENP* expression is associated with shorter survival if either hormone therapy or chemotherapy were given. However, further data are needed to confirm the impact of *INCENP* expression on the response to chemotherapy in luminal BC.

At the mRNA levels, we detected a significant correlation between high *INCENP* expression and adverse clinical, and pathological characteristics and short patient survival. High *INCENP* mRNA predicted poor outcomes in luminal and TNBC tumours. This may imply that *INCENP* plays a role in tumorigenic pathways and could be a marker of poor prognosis in both luminal and TNBC. Our findings are in line with those of Sun et al, who showed that alterations in *INCENP* mRNA are linked to a poor prognosis in neuroblastoma patients [11].

The discrepancy between *INCENP* protein and mRNA regarding the prognostic significance in TNBC might be attributed to the differences in the number of cases in each subtype between the Nottingham and METABRIC cohorts or might be due to tumour-specific differences in *INCENP* mRNA/protein stability or post-transcriptional regulation of *INCENP* expression, or redistribution from the nucleus into the cytoplasm during metaphase anaphase transition. Based on these findings, *INCENP* might be

used as an additional progression/transformation marker in luminal and TNBC. We reported a higher percentage of *INCENP* expression at the proteomic level compared to the mRNA level. This could be related to the cut-off of positivity used and the sensitivity of the IHC technique used.

This study has some limitations. The subjectivity of the semi-quantitative H-score method, that has been used to score the sections, is one of our study weaknesses. To reduce the impact of this limitation 20% of the cores were rescored to ensure the reproducibility and liability of the procedure. This study has been performed on TMA, which would underestimate the role of intratumor heterogeneity. However, all cases in our cohort were histologically reviewed prior to the construction of the TMA and multiple cores for cases with heterogeneous grades or morphological patterns have been used to represent various tumour areas. The large number of patients used in this study can compensate, statistically, for the potential heterogeneous *INCENP* expression within the tumour. Also, the small number of full-face stained sections showed homogenous staining throughout the tumour and sparing the surrounding stroma.

In conclusion, the expression and subcellular localisation of INCENP expression appears to play a role in BC progression. High nuclear INCENP expression is related to aggressive types and poor outcomes in BC. Further functional studies of INCENP in BC with consideration of its subcellular localisation in tumour cells are warranted. *INCENP* was associated with poor prognostic characteristics and poor survival outcomes. Overexpression of *INCENP* appears to play a role in the progression of Luminal and TNBC and thus, it could act as a potential prognostic marker and a therapeutic target. Functional assessment is warranted to reveal the specific role played by *INCENP* in BC.

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Statement of Ethics This study was approved by the Nottingham Research Ethics Committee 2 under the title 'Development of a molecular genetic classification of breast cancer and by the North West – Greater Manchester Central Research Ethics Committee under the title 'Nottingham Health Science Biobank (NHSB)' reference number 15/ NW/0685. Written informed consent was obtained from all individuals prior to surgery to use their tissue materials in research.

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Authors contribution: AI scored all the cases, took the lead in writing the manuscript, data analysis and interpretation, IMM and AG helped in data interpretation and reviewing the article. MT contributed to data analysis, study design and reviewing the article. EAR: conceived and planned the presented idea, data interpretation and reviewing the article.

Data Availability Statement: All data generated or analysed during this study are included in this article. Further enquiries can be directed to the corresponding author.

Conflict of Interest Statement

All the authors declare that they have no conflict of interest.

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Figure Legends:

Figure (1): Immunohistochemical analysis of the morphological characteristics of INCENP in full face sections.

A. & B. Nuclear and cytoplasmic immunoreactivity of INCENP in invasive breast cancer cells was stronger than that observed in normal epithelial cells. (Magnification: × 200).

B. The normal terminal duct-lobular unit showed negative immunoreactivity of INCENP (magnification: × 200).

C. The expression of INCENP in the stromal cells was weak or negative (magnification: × 200).

Figure (2): INCENP expression in different mitotic phases

Detection of INCENP in all stages of normal and abnormal mitoses.

A. prophase, **B.** metaphase, **C.** anaphase, **D.** abnormal-multipolar mitotic figure. (magnification: × 400).

Figure (3): INCENP TMA protein expression, A &B: Negative INCENP IHC expression**, D, E & F:** Positive INCENP IHC nuclear expression in invasive breast cancer TMA cores.

Figure (4): Association between INCENP nuclear expression and patient outcome of invasive BC

INCENP nuclear expression against breast-cancer-specific survival (BCSS) in **A**. All cases, **B**. Luminal A tumors **C**. Luminal B tumor, **D**. Triple negative breast cancer (TNBC). **E**. Human epidermal growth factor receptor 2 (HER2 +) tumors. And INCENP nuclear expression and distant metastasis free survival (DMFS) in **F**. All cases, **G**. Luminal A tumors, **H.** Luminal B tumors, **I.** Triple negative breast cancer

(TNBC). **J**. Human epidermal growth factor receptor 2 (HER2 +) tumors, in the Nottingham cohort.

Figure (5): Kaplan–Meier survival plots showing the association between INCENP nuclear expression and breast cancer specific survival (BCSS) in **A.** Chemotherapy treated patients, **B.** Hormonal therapy treated patients. Similarly, the association between INCENP nuclear expression and distant metastasis free survival (DMFS) in **C.** Chemotherapy treated patients, and **D.** Hormonal therapy-treated patients.