KIF21A influences breast cancer metastasis and survival

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ABSTRACT

Breast cancer pathogenesis is known to be propagated by the differential expression of a group of proteins called the Kinesin Superfamily (KIFs), which are instrumental in the intracellular transport of chromosomes along microtubules during mitosis. During mitosis, KIFs are strictly regulated through temporal synthesis so that they are only present when needed. However, their misregulation may contribute to uncontrolled cell growth due to premature sister chromatid separation, highlighting their involvement in tumorigenesis. One particular KIF, KIF21A, was recently found to promote the survival of human breast cancer cells *in vitro*. However, how KIF21A influences other cancerous phenotypes is currently unknown. This study therefore aimed to consolidate the *in vitro* role of KIF21A in breast cancer metastasis, while also analysing KIF21A expression in human breast cancer tissue to determine its prognostic value. This was achieved by silencing KIF21A in MCF-7 and MDA-

MB-231 breast cancer cell lines via siRNA transfection. Migration, invasion, proliferation, and adhesion assays were then performed to measure the effects of KIF21A silencing on oncogenic behaviour. Immunohistochemistry was also conducted in 263 breast cancer tissue samples to compare KIF21A expression levels against various prognostic outcomes and clinicopathological parameters. KIF21A knockdown reduced cell migration (by 42.8% [MCF-7] and 69.7% [MDA-MB-231]) and invasion (by 72.5% [MCF-7] and 42.5% [MDA-MB-231]) in both cell lines, but had no effect on adhesion or proliferation, suggesting that KIF21A plays an important role in the early stages of breast cancer metastasis. Unexpectedly however, KIF21A was shown to negatively correlate with various pro-malignant clinicopathological parameters, including tumour size and histological grade, and high KIF21A expression predicted better breast cancer survival (hazard ratio = 0.45), suggesting that KIF21A is a tumour suppressor. The conflicting outcomes of *in vitro* and *in vivo* data may be due to the possible multi-functionality of KIF21A or study limitations, and means no definitive conclusions can be drawn about the role of KIF21A in breast cancer. This warrants further investigation, which may prove pivotal to the development of novel chemotherapeutic strategies to mediate KIF21A's function and enhance prognostic outcomes.

1. INTRODUCTION

Breast cancer is the most frequently diagnosed cancer among women [1], and its metastasis often leads to death in patients. Breast cancer pathogenesis is known to be affected by the differential expression of a group of proteins called the kinesin superfamily (KIF) [2].

Kinesins were first isolated from squid tissue and identified as molecular motors for axonal transport that are ubiquitous in all eukaryotes [3]. In total, 45 kinesins have been identified in humans and other mammals [4,5]. They are sorted into 14 subfamilies based on structural

differences, however all share a highly-conserved motor domain that provides motor binding to microtubules [6]. They have adenosine triphosphate (ATP) activity and microtubule-dependent motion potential, allowing movement along microtubules through coupling energy from ATP hydrolysis to force production [6].

The role of kinesins has since been specified in the transport of vesicles and organelles within cells, and chromosomes during mitosis and meiosis. During mitosis, kinesins are strictly regulated through temporal synthesis so that they are only present when needed [7]. However, misregulation of kinesins during the cell cycle may contribute to uncontrolled cell growth, highlighting their involvement in tumorigenesis. For example, *either* the depletion or overexpression of some mitotic kinesins can lead to unbalanced movement of chromosomes along microtubules during mitosis [8]. This causes a cascade of excessive spindle separation, premature sister chromatid separation, overshooting before anaphase, and finally unequal distribution of DNA and aneuploidy [9-11]. The aneuploid daughter cells may display cancerous behaviour, including increased metastatic behaviour [12].

Furthermore, Some KIFs have recently been associated with poor prognosis [13-18] and chemotherapeutic drug resistance [19-21] in breast cancer patients and cell lines, respectively. Such an involvement in mitotic deregulation, clinical outcomes and chemotherapeutic resistance in breast cancer highlights the importance of understanding the molecular mechanisms underlying the behaviour of KIFs.

One particular KIF, KIF21A, was recently found to promote lysosomal stability and the survival of human breast cancer cells *in vitro* [22]. However, how KIF21A distinctly affects other malignant phenotypes and prognostic outcomes in breast cancer is currently unknown. This study therefore aimed to (1) identify whether KIF21A is overexpressed in breast cancer tissue; (2) understand the functional behaviour of KIF21A in breast cancer cell lines and (3)

evaluate the associations between KIF21A expression levels and breast cancer recurrence, survival and various clinicopathological parameters. KIF21A functional behaviour was assessed via migration, invasion, proliferation, and adhesion assays in KIF21A-silenced versus wildtype breast cancer cell lines. Immunohistochemistry staining of KIF21A in clinical IDC breast tissue microarrays was then performed to determine KIF21A expression in breast cancer tissue and to examine relationships between KIF21A expression, clinicopathological data and prognostic outcomes. We hypothesised that KIF21A shows upregulated expression in breast cancer tissue and correlates with poor prognostic outcomes, and KIF21A enhances pro-cancerous phenotypes *in vitro*, including migration, invasion, proliferation and adhesion. Identifying exactly how KIF21A is involved in breast carcinogenesis may prove pivotal to the development of chemotherapeutic targeting to mediate its function and improve prognostic outcomes.

2. MATERIALS AND METHODS

2.1 Cell Culture

MCF-7 (ATCC: HTB-22, VA, USA) and MDA-MB-231 (ATCC: HTB-2) cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) and Roswell Park Memorial Institute medium 1640 (RPMI 1640) (Hyclone) supplemented with 10% FBS, respectively. Both cell lines were incubated at 37 °C in a humidified incubator with 5% CO₂. All cell culture was done without antibiotics.

2.2 Small Interfering RNA (siRNA) Transfection

Conditions used to achieve favourable silencing efficiency in both cell lines were as follows: 1 x 10⁵ (MCF-7) or 2 x 10⁵ (MDA-MB-231) cells per well were seeded with culture medium in a 6-well plate and incubated for 24 hours, which enabled the cells to reach at least 30% confluence. Transfection with Ambion Silencer Select siRNA (Ambion, Foster City, CA, USA; Table 1) was performed using Oligofectamine (Thermo Fisher Scientific, Wilmington, DE, USA) in Opti-MEM1 Reduced Serum Medium (Thermo Fisher Scientific). For MCF-7 cells, 10 µL siRNA was mixed with 175 µL Opti-MEM1 in one tube, while 5 µL Oligofectamine was mixed with 10 µL Opti-MEM1 in another tube. For MDA-MB-231 cells, 10 µL siRNA was mixed with 100 µL Opti-MEM1 in one tube, while 10 µL Oligofectamine was mixed with 100 µL Opti-MEM1 in another tube. The tubes were incubated at room temperature for five minutes, after which the contents of both were mixed and incubated again at room temperature for a further 20 minutes. The medium in each well of the 6-well plate was removed and replaced with 800 µL (MCF-7) or 780 µL (MDA-MB-231) Opti-MEM1 and 200 µL (MCF-7) or 220 µL (MDA-MB-231) of the previously-incubated siRNA-Oligofectamine mixture. The plate was then left to incubate for 8 hours at 37 °C in a humidified incubator with 5% CO₂. 500 µL culture medium (30% FBS) was then added to each well. At 24 hours post-transfection, the medium was replaced with fresh medium (supplemented with 10% FBS). At 48 hours post-transfection, cells were harvested from each well for use in subsequent experiments. Ambion Silencer Select Scrambled siRNA was used as the negative control and Ambion Silencer Select GAPDH siRNA was used as the positive control.

2.3 RNA Extraction and One-step qRT-PCR

Total RNA was extracted using the Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) according to manufacturer's instructions. RNA yield and purity were then

quantified using the Nanodrop ND-100 Spectrophotometer (Thermo Fisher Scientific) according to manufacturer's protocol. Extracted RNA was subjected to one-step qRT-PCR using the iTaq Universal SYBR Green One-Step Kit (Bio-Rad, Hercules, CA, USA), following manufacturer's instructions, on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The qRT-PCR primers used for this study (1st BASE, Singapore) are shown in Table 2. The program settings used for qRT-PCR were: (1) reverse transcription at 50 °C for 10 minutes; (2) activation step at 95 °C for 30 seconds; (3) 45 cycles of denaturation at 95 °C for 5 seconds and annealing at 60 °C for 30 seconds; and (4) melt curve analysis.

2.4 Western Blotting

Protein was isolated from cells 72 hours post-transfection using M-PER Mammalian Protein Extraction Reagent (200 µL/well) (Thermo Fisher Scientific) mixed with 10 µg/mL Halt Protease Inhibitor Cocktail (2 µL/well) and EDTA (2 µL/well) (Thermo Fisher Scientific). The buffer mixture was added to cells and incubated for 5 minutes on ice. Cells were then scraped off with a cell scraper (TPP, Trasadingen, Switzerland), aspirated, and centrifuged at 16,000 g for 10 minutes at 4 °C. The resulting supernatant was extracted and stored at -80 °C. Following extraction, protein samples were quantified using the Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Fisher Scientific) according to manufacturer's protocol and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Following the SDS-PAGE run, protein samples were transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) via the wet transfer method. Protein was subsequently transferred at 100 V for 1 hour at 4 °C. The membrane was then blocked with 5% BSA (Sigma-Aldrich) in 1X Tris-Buffered Saline and 1% Tween 20 (TBST) for 2 hours, and incubated at 4 °C overnight with primary antibodies (Table 4.3) that

were diluted in 5% BSA. Primary antibodies were then removed and the membrane was washed with 1X TBST three times for 10 minutes each time. Secondary antibodies (Table 4.3) were then added and incubated for 1 hour at room temperature. After which, the membrane was washed with 1X TBST three times for 10 minutes each time.

2.5 Transwell Migration Assay

Migration assays were performed using Costar 6.5 mm Transwell chambers with 8.0 μ m pore polycarbonate membrane inserts (Corning, Lowell, MA, USA). siRNA transfection was performed as per section 4.2. 48 hours post-transfection, cells were harvested, resuspended in fresh medium (10% FBS), and 5×10^4 (MCF-7) or 3×10^4 (MDA-MB-231) cells were seeded into the inserts. The medium outside the inserts was 600 μ L fresh medium supplemented with 30% FBS. The plate was then left in an incubator for 24 hours at 37 °C. Cell suspension was then removed from inside the inserts. Migrated cells on the outer surface of the membrane of the inserts were subsequently fixed by 100% methanol and stained with 0.5% (w/v) crystal violet indicator (Sigma-Aldrich). Visualisation of the migrated cells was performed using a 10X objective lens under a Nikon SMZ1500 stereomicroscope coupled to a Nikon DXM1200F digital camera (Nikon, Minato, Tokyo, Japan). Five random field images per insert were captured for quantification.

2.6 Matrigel Invasion Assay

The invasion assay was performed in a similar manner to the migration assay, although the chambers used were BD BioCoat Matrigel Invasion Chambers with 8.0 μ m pore size inserts (BD Biosciences, San Jose, CA, USA). siRNA transfection was performed as per section 4.2. 48 hours post-transfection, cells were harvested, resuspended in fresh medium (10% FBS), and 1×10^5 (MCF-7) or 5×10^4 (MDA-MB-231) cells were seeded into the inserts. The

medium on the outside of the inserts was 600 μ L fresh culture medium, supplemented with 30% FBS. The plate was left in an incubator for 24 hours at 37 °C. Invaded cells were then fixed, stained, imaged and quantified in an identical manner to the migration assay.

2.7 Serum-Starved Proliferation Assay

Proliferation assays were performed using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Fitchburg, WI, USA). To begin, cells were serum-starved overnight prior to seeding, i.e. sub-cultured in fresh medium in a 25-cm² flask without FBS. siRNA transfection was performed as per section 4.2. 72 hours post-transfection, culture medium was removed and replaced with 1.8 mL fresh medium (10% FBS) with 300 μ L MTS reagent. Samples were incubated at 37 °C for 1 hour prior to measuring absorbance values once every hour for 4 hours. Formazan absorbance was detected at 490 nm by a GENios Plate Reader (Tecan, Austria).

2.8 Cell Adhesion Assay

96-well plates were coated overnight with either 50 μ L collagen I (Corning) or 50 μ L fibronectin (BD Biosciences), both at concentrations of 20 μ g/mL. Collagen I and fibronectin were then removed and the wells were washed twice with 1X PBS. 100 μ L 1% BSA (Sigma-Aldrich) was then added to the same wells for 1 hour at room temperature for blocking. BSA was then removed and the wells were washed twice with 1X PBS. siRNA transfection was performed as per section 4.2. 48 hours post-transfection, cells were harvested, resuspended with fresh medium and seeded into the wells at a density of 1 x 10⁴ cells for collagen I and 3 x 10⁴ cells for fibronectin wells. The 96-well plate was then incubated for 60 minutes at 37 °C for cells to adhere. Subsequently, non-adhered cells were washed off by 1X PBS. 100 μ L complete medium and 20 μ L MTS were then added to each well. Formazan absorbance was detected at 490 nm by a GENios Plate Reader (Tecan) once every hour for 4 hours.

2.9 Immunohistochemistry (IHC)

Archived, formalin-fixed, paraffin-embedded breast cancer tissue samples were received from the Department of Pathology, Singapore General Hospital (SGH). Only breast invasive ductal carcinoma (IDC) cases were used for this study. A total of 287 breast IDC cases from 1997 to 2007 were analysed, including 263 tumour tissue samples and 24 adjacent normal counterparts (all female). For the full distribution of clinicopathological data see Table 4. The collection of human tissue samples for this study received ethical approval from the Institutional Review Board, Singapore General Hospital.

Slides were prepared using tissue microarray (TMA) technology. The TMA slides were deparaffinised twice in Clearene (Leica Biosystems), rehydrated in a graded series of ethanol, and finally washed in distilled water. The slides were then washed in 1X Tris-buffered saline (TBS) (Bio-Rad), incubated with 3% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity, and washed in 1X TBS with 1% Triton X-100 (TBS-TX) (Bio-Rad) thrice. Antigen retrieval was performed by boiling the slides at 100 °C for 20 minutes in a 500 mL solution containing 0.1 M sodium citrate, 0.1 M citric acid and distilled water. After cooling to room temperature, the slides were washed in 1X TBS-TX three times. They were then blocked for 1 hour using 1:100 dilution goat serum (Dako, Agilent Technologies, Denmark) at room temperature, followed by KIF21A primary antibody (1:100 dilution; Table 2) incubation overnight at 4 °C. The next day, the slides were washed in 1X TBS-TX, then incubated with undiluted horseradish peroxidase (HRP) conjugated secondary antibodies (polyclonal goat anti-rabbit immunoglobulins, Dako, catalogue no. K4010) for 1 hour at room temperature, and washed again in 1X TBS-TX. To visualise the staining, the slides were incubated with 3,3'-Diaminobenzidine (DAB) (Thermo Fisher Scientific) for 30

minutes, followed by counter-staining in filtered concentrated Shandon Harris haematoxylin (Thermo Fisher Scientific) for 30 seconds. The slides were finally dehydrated in a graded series of ethanol and washed twice in Clearene, followed by fixing in Permount (Thermo Fisher Scientific).

The fixed TMA slides were scanned and their staining intensities assessed using Philips Image Management System software (Philips, Amsterdam, Netherlands). KIF21A immunopositivity was scored independently by one individual followed by confirmation by a trained pathologist. The scoring criteria was based on KIF21A staining intensities with '0' = no staining, '1+' = weak staining intensity, '2+' = moderate staining intensity, and '3+' = strong staining intensity. Weighted average intensity (WAI) was then calculated, which is the average intensity of each stained cell

2.10 Statistical Analysis

For qRT-PCR and all functional assays, Prism 5 (GraphPad Software, La Jolla, CA, USA) was used to conduct statistical analysis. One-way ANOVAs were utilised to detect significant differences ($p < 0.05$) between wildtype cells and two groups of KIF21A-silenced cells (each group used a different siRNA sequence), followed by Tukey's multiple comparisons post-hoc test. Immunohistochemistry results were analysed using SPSS 18.0 for Windows (SPSS Inc., Chicago, IL, USA). KIF21A immunostaining in malignant versus normal tissues were compared using the non-parametric Mann-Whitney test. Relationships between KIF21A immunoscores and nominal clinicopathological parameters were analysed using Fisher's exact test, while relationships with ordinal parameters were analysed using Kendall's tau-c test. For the survival analyses (recurrence and mortality), the timeline measures (in months) were: (1) OS (Overall survival) = Date of Death – Date of Diagnosis; (2) SAR (Survival after

Recurrence) = Date of Death – Date of Recurrence; (3) DFS (Disease free survival) = Date of Recurrence – Date of Diagnosis. Survival analyses were performed using Kaplan-Meier analysis and the log-rank (Mantel-Cox) test, and variables that achieved statistical significance in univariate analyses ($p < 0.05$) were subsequently entered into a multivariate analysis using the Cox proportional hazards model via the backward stepwise regression method (Model A). In addition, to examine the predictive value of KIF21A expression in greater detail, the analysis included a multivariate Cox proportional hazards model that included *all* clinicopathological parameters via the enter method (Model B; Table 5.5). Statistical significance was set at $p < 0.05$ for all tests.

3. RESULTS

3.1 KIF21A Silencing in Breast Cancer Cell Lines

qRT-PCR analysis showed KIF21A was significantly silenced using two different siRNA sequences, siKIF21A-1 and siKIF21A-2 in MCF-7 cells by 39.8% ($p < 0.01$) and 73.8% ($p < 0.001$), respectively, and in MDA-MB-231 cells by 67.8% ($p < 0.001$) and 62.7% ($p < 0.01$), respectively (Figures 1A,B). Western blot analysis showed silencing via siKIF21A-1 and siKIF21A-2 translated to a reduction in protein levels in MCF-7 cells by 41.8% ($p < 0.05$) and 66.4% ($p < 0.01$), respectively, and in MDA-MB-231 cells by 58.3% ($p < 0.05$) and 69.7% ($p < 0.01$), respectively (Figures 1 C,D).

3.2 Transwell Migration Assay

KIF21A silencing in MCF-7 resulted in a 42.8% ($p < 0.01$) and 39.8% ($p < 0.01$) decrease in the average number of migrated cells for siKIF21A-1 and siKIF21A-2 groups, respectively, compared to the negative control (Figures 2A,B). KIF21A silencing in MDA-MB-231 reduced cell migration by 69.7% ($p < 0.0001$) and 64.6% ($p < 0.001$) for siKIF21A-1 and

siKIF21A-2, respectively, compared to the negative control (Figures 2C,D).

3.3 Matrigel Invasion Assay

KIF21A silencing in MCF-7 resulted in a 42.5% ($p < 0.001$) and 72.5% ($p < 0.0001$) decrease in the average number of invaded cells for siKIF21A-1 and siKIF21A-2 groups, respectively, compared to the negative control (Figures 3A,B). KIF21A silencing in MDA-MB-231 reduced cell invasion by 42.5% ($p < 0.05$) and 41.0% ($p < 0.05$) for siKIF21A-1 and siKIF21A-2, respectively, compared to the negative control (Figures 3C,D).

3.4 Cell Proliferation Assay

Analysis of cell proliferation showed no significant differences in cell proliferation between the negative and KIF21A-silenced groups for both MCF-7 and MDA-MB-231 cells (Figures 4A,B).

3.5 Cell Adhesion Assay

For MCF-7 cells, analysis showed no significant differences in cell adhesion to both collagen I and fibronectin between the negative and KIF21A-silenced groups (Figures 4C,D). For MDA-MB-231 cells, KIF21A silencing also had no significant effect on cell adhesion to collagen I (Figure 4E). Interestingly, however, silenced MDA-MB-231 cells displayed reduced adhesion to fibronectin by 47.2% ($p < 0.05$) and 54.0% ($p < 0.05$) for siKIF21A-1 and siKIF21A-2, respectively (Figure 4F).

3.6 Comparison of KIF21A Staining Between Normal and Tumorous Breast IDC Tissues

KIF21A staining was predominately observed in the nucleus of both normal and tumorous breast epithelial cells, while cytoplasmic staining was either absent or minimal (Figure

5A,B,C). Analysis showed no significant differences in KIF21A nuclear staining between tumour and normal samples (Figure 5D).

3.7 Relationship Between KIF21A Expression and Clinicopathological Parameters in Breast IDC Tissues

Cut-off values above and below the mean WAI (1.0) were determined as “high” and “low” KIF21A expression, respectively. High KIF21A expression correlated with tumour size below 20 mm ($p < 0.05$), and progressively lower histological grades ($p < 0.01$; Table 5). No significant correlations were observed between KIF21A and other parameters.

3.8 Univariate (Kaplan-Meier) analysis of survival and KIF21A expression

The three timelines investigated were overall survival (OS), survival after recurrence (SAR), and disease-free survival (DFS). Survival data is cause-specific and was available for all cases, with a follow-up period ranging from 0 months to 156 months. It should be noted that no patients survived after breast cancer recurrence for the entire follow-up period (Table 6). Surprisingly, Kaplan-Meier analysis showed patients with high nuclear KIF21A expression were more likely to have better overall survival ($p < 0.05$; Figure 6A) and survival after recurrence ($p < 0.05$; Figure 6B). However, nuclear KIF21A expression was not identified as a significant predictor of breast cancer recurrence, as measured by DFS (Figure 6C).

3.9 Multivariate (Cox proportional hazards regression) analysis of survival and KIF21A expression

Variables that achieved statistical significance in univariate analyses ($p < 0.05$) were subsequently entered into a multivariate analysis using Cox proportional hazards model via the backward stepwise regression method (Model A; Table 7). In addition, to examine the

prognostic value of KIF21A in greater detail, the analysis included a multivariate Cox proportional hazards model that included *all* clinicopathological parameters via the enter method (Model B; Table 7). This included patients' age, race, tumour size, histological grade, lymph node involvement, lymphovascular invasion, and ER, PR and HER2 statuses. However, only those which showed statistical significance ($p < 0.05$) are shown in Table 7. Model A showed KIF21A acts independently to predict SAR ($p < 0.05$), but significantly correlates with tumour size in predicting OS ($p < 0.05$). Model B predicted KIF21A as an independent prognostic factor for both OS ($p < 0.05$) and SAR ($p < 0.05$). However, both models showed KIF21A expression was not a prognostic factor for recurrence, as measured by DFS.

4. DISCUSSION

4.1 KIF21A Mediates Breast Cancer Metastasis *In Vitro*

This study identifies KIF21A as a protein whose depletion consistently caused a statistically and biologically significant reduction in breast cancer cell migration and invasion *in vitro* (Figures 2 and 3). To our knowledge, this is the first study to identify the role of KIF21A in cancer cell metastasis – an important finding in the assessment of KIF21A as a potential therapeutic target. Invasion of malignant cells into the surrounding ECM and migration of invaded cells towards the bloodstream are pivotal early steps in metastasis, suggesting that KIF21A is an important mediator of early carcinogenesis. The observed migratory influence of KIF21A was enhanced in MDA-MB-231 cells, which are known to be highly metastatic, while higher silencing efficiency using siKIF21A-2 in MCF-7 intensified the reduction in migration. Furthermore, although a more optimal silencing efficiency would be preferred to observe the full effects of KIF21A knockdown, there was still a significant reduction in both

invasion and migration. Whether complete knockdown would have accentuated this observation remains unknown. Nonetheless, these cumulative findings suggest that KIF21A may be a key component of breast cancer cell migratory and invasive pathways. This could be explained by its interaction with other molecules and their known mechanisms (see Section 4.3 below).

However, these observations surprisingly contradicted the *in vivo* findings of KIF21A expression in human breast cancer tissue (discussed in-detail in Section 4.2), meaning that KIF21A's role in cancer metastasis is largely inconclusive. Despite this, *in vitro* functional assays do serve an important purpose. Although they are only representative of complex *in vivo* conditions, they provide a critical understanding about the influence of genes on distinct metastatic components. In this case, KIF21A was shown to influence the distinct metastatic components of migration and invasion – a phenomenon supported by studies of other kinesin family members. KIF3A and KIF3B, for example, have been shown to interact in a complex that transports proteins essential for cancer cell migration [23], while KIF11 has been found to respond to directional cues during chemotaxis to govern the direction of MDA-MB-231 cell migration [24]. Other studies identify KIF14 [25], KIF2A [26], and KIF18A [27] as mediators of breast cancer migration *in vitro* via a range of pathways. Given the discrepant results of the current study, migration and invasion assays should be extended to *in vivo* models to elucidate KIF21A's precise role in these processes.

Following migration, cancer cells must adhere to the ECM of a new site where they can then rapidly proliferate. This study investigated the role of KIF21A in this process. KIF21A knockdown by 40-70% did not induce any changes in cell proliferation for both breast cancer cell lines (Figure 4), and thus KIF21A is not implicated as a player in breast cell proliferation. Although it is difficult to identify studies reporting similarly negative results for

other kinesin family members, the present study shows KIF21A varies from previous reports of KIFs acting as key mediators of proliferation in breast cancer [26,28].

KIF21A was also tested for cell adhesion to collagen I and fibronectin fibers – both protein components of the ECM – yet no changes were observed following silencing in MCF-7 for both fibers, and in MDA-MB-231 for collagen I fibers (Figure 4). However, reduced adhesion to fibronectin did occur in MDA-MB-231 cells, suggesting KIF21A could facilitate breast cancer cell establishment in foreign sites, particularly for highly invasive cells. The fundamental genetic differences between MCF-7 and MDA-MB-231 cells may have contributed to differences with their adhesion assays. Further analysis is thus required to investigate the functional role of KIF21A in breast cancer cell adhesion, which could be done via overexpression studies and other adhesion assays involving laminin, gelatin and collagen II fibers. More broadly, there is no particular commonality in the adhesive capabilities of other kinesins, with many being shown to either positively or negatively regulate adhesion in various cell lines [25,29,30]. Further studies in KIF21A are therefore encouraged.

4.2 Disparity Between *In Vitro* and *In Vivo* Results

The present study suggested KIF21A exerts oncogenic activity *in vitro*, which resonates with our hypothesis and previous studies that show KIF21A facilitates the survival of breast cancer cell lines [22]. However, conflicting results were obtained *in vivo*. Immunohistochemical analysis suggested KIF21A exerts tumour-suppressive functions, rather than oncogenic activity. Although it is unclear why different results were obtained for the same gene, there are several explanations for this phenomenon: (1) ethnic differences. Various studies illustrate that the mutation status of numerous genes contributing to breast cancer is different between Asian and Caucasian populations. This study was predominately performed in Asian individuals, while MCF-7 cells and MDA-MB-231 cells were isolated

from Caucasian women. Ethnicity may therefore be a contributing factor to any discrepancies, and *in vivo* studies of KIF21A in Caucasian populations are encouraged. (2) One molecule may have dual or multiple physiological functions. For example, both transforming growth factor beta (TGF-beta) and signal transducer and activator of transcription 3 (STAT3) have oncogenic or tumour-suppressive roles depending on different conditions including the mutational background of the tumor [31-33]. Whether KIF21A has a dual effect remains unknown, however similarly conflicting studies have been observed in other kinesins, particularly KIF14, which has been shown to both promote and suppress tumorigenesis [15,34]. (3) Study limitations. *In vitro* assays are useful in studying the influence of various molecules on distinct metastatic components, but we must not forget the amazingly complex story of the tumour microenvironment. Firstly, despite strict conditions, cell line studies cannot control for the vast array of biological parameters that would otherwise be present *in vivo*. Cell lines studies are therefore better used as tools to delineate the molecular events underpinning carcinogenesis rather than predicting the entire metastatic cascade. Secondly, cell lines are only representative of one individual. Although two breast cancer cell lines were used for support in this study, they cannot match the statistical power of the 263 breast IDC patient samples analysed via IHC. Of course, some patients may have indeed had a poorer prognosis with high KIF21A expression, which would resonate with the *in vitro* findings. However, there was a significant trend towards poorer prognosis correlating with low KIF21A expression. Further *in vitro* analyses are therefore encouraged in more breast cancer cell lines that are subject to both KIF21A silencing and overexpression.

4.3 KIF21A Signaling Pathways Leading to Cell Migration and Invasion

Although few studies have investigated KIF21A beyond its role in congenital fibrosis of the extraocular muscles type-1 (CFEOM1), evidence suggests it may interact with other genes

involved in cell migration. KIF21A binds to brefeldin A (BFA)-inhibited guanine nucleotide-exchange factor (BIG1) [35] to maintain the organisation of the Golgi apparatus [36], and also transports KN motif and ankyrin repeat domains 1 (KANK1), influencing its membrane localisation [37]. Subsequent exploration revealed that both BIG1 and KANK1 co-immunoprecipitated with KIF21A and each other, such that they may act as a scaffold in KIF21A-powered intracellular transport [38]. The interaction between all three molecules was shown to affect cell migration; a phenomenon explained by their combined effects on cell polarity. Generation and maintenance of cell polarity is essential for directional migration and results from asymmetric membrane traffic achieved by intracellular transport and the delivery of extra membrane to the cell's leading edge [39,40]. KIF21A is theorised to act via BIG1 and KANK1 to position the Golgi and microtubule-organising center (MTOC) structures anterior to the nucleus, resulting in a polarity shift that induces front-end-directed cell migration [38]. This interaction would explain the observations seen in breast cancer cell migration in the present study. In support of this theory, previous studies show polarisation of Golgi and MTOC structures to be explicitly disturbed by KANK1 or BIG1 siRNA treatment [38]. However, further investigation is required to identify additional players in KIF21A/BIG1/KANK1 functional interactions and to elucidate the detailed molecular mechanisms that alter cell polarity to influence directional migration.

4.4 KIF21A in Breast Cancer Tissues

Although our *in vitro* data strongly supported a tumorigenic role for KIF21A in breast cancer, IHC in breast cancer tissue microarrays was contrary to our hypothesis and showed KIF21A associated with anti-malignant phenotypes and a better prognosis. Both analyses suggest KIF21A is involved in breast cancer pathogenesis and prognosis, however their conflicting outcomes mean the overall results of this study remain largely inconclusive.

Nonetheless, numerous important findings came from the IHC analysis. Firstly, there were no significant differences in KIF21A expression between breast IDC samples and adjacent normal tissues (Figure 5). This adds to inconsistent evidence regarding the role of KIF21A in breast oncogenesis, and is contradictory to previous studies in the kinesin superfamily, which observe upregulation of numerous KIFs in breast cancer [13,15,17]. KIF21A may therefore not be a useful diagnostic biomarker.

In addition, KIF21A expression was shown to negatively correlate with the pro-malignant phenotypes of large tumour size (≥ 20 mm) and high histological grade (Table 5). Large tumour size and high histological grade classically predict poorer prognosis in breast cancer [41], so KIF21A may exert a tumour-suppressive role during breast carcinogenesis. However, the mechanism via which it performs this role was not clear from the IHC analysis, as KIF21A displayed no relationships with lymphovascular invasion, lymph node involvement, or various receptors including ER, PR, and HER2. This suggests KIF21A may not regulate distant metastasis to lymph nodes and blood vessels (in contradiction to the *in vitro* observations), and it may not be involved in the mechanisms through which oestrogen, progesterone and human epidermal growth factor increase breast cell proliferation. This differs from previous observations of concerted KIF21A upregulation upon the introduction of exogenous oestrogen *in vitro* [42]. One explanation for KIF21A-induced tumour suppression may therefore be its role in mitosis (see Section 4.5 below).

Survival analyses were also performed, and revealed high KIF21A expression predicted better OS and SAR, but had no significant associations with DFS (Figure 6). KIF21A may therefore act as a tumour suppressor, and may be used as a predictor of breast cancer survival, but not recurrence. To identify whether KIF21A predicted survival independently of

other clinicopathological parameters, multivariate analyses were also performed (Table 7). Although univariate analysis suggested KIF21A co-predicted OS and SAR with tumour size and histological grade, Cox proportional hazards models revealed KIF21A acted independently. KIF21A may therefore be an independent prognostic biomarker for better breast cancer survival. In this study cohort, lymphovascular invasion, lymph node involvement, and hormonal and growth factor receptor statuses had no correlations with SAR and OS – a rare finding (although some of those variables did correlate with recurrence). Nonetheless, this observation adds to evidence that KIF21A functions independently of lymphovascular invasive pathways, oestrogen, progesterone, and human epidermal growth factor to implement its tumour-suppressive activity.

The possible role of KIF21A as a tumour suppressor and predictor of better breast cancer prognosis differs from that of most other kinesin family members. KIFs 2A [43], 2C [17], 3C [44], and 26B [45] have all been identified as predictors of worse breast cancer prognosis. Intriguingly however, a select few kinesins have been observed as tumour-suppressor genes consistent with the results of the current study, albeit in other cancer types. Overexpression of both KIF4 [46] and KIF14 [34] has been shown to reduce metastatic phenotypes *in vitro* and predict better survival outcomes in human gastric carcinoma and lung adenocarcinoma, respectively. In both cases, it was theorised that they carried out their tumour-suppressive functions through their roles in mitosis – a phenomenon that could explain similar observations in this study (see below).

4.5 Mitotic Misregulation as a Functional Explanation for KIF21A-mediated Tumorigenesis

Errors at any point during the cell cycle can be catastrophic and in humans can lead to cancer

[47]. Chromosome mis-segregation during mitosis, for example, results in abnormal cytokinesis and aneuploidy [48], which is cleared in normal cells through apoptosis. However, tumour cells show higher rates of aneuploidy, which is often associated with poor clinical outcomes [49-51]. Interestingly, misregulation of various mitotic kinesins has been shown to result in aneuploidy, because unbalanced movement of kinesins can cause excessive spindle separation, premature sister chromatid detachment, overshooting before anaphase, and eventually unequal distribution of DNA [8-11]. The aneuploid daughter cells could, theoretically, display any possible tumorigenic phenotype and a plethora of metastatic characteristics, including aberrant migration and invasion. Via this mechanism, KIF21A depletion could result in aneuploidy to facilitate tumour formation, which explains the *in vivo* findings of this study. High KIF21A expression may therefore suppress carcinogenesis if KIF21A is implicated in mitosis.

4.6 Future Work

The results of this study were largely inconclusive due to discrepancies between *in vitro* and clinical data. To further elucidate KIF21A's role in breast cancer, other functional analyses should therefore be performed (e.g. apoptosis and drug resistance assays) in more breast cancer cell lines that have been subjected to KIF21A knockdown, overexpression and the introduction of exogenous KIF21A. This would solidify knowledge about KIF21A's role in breast cancer *in vitro*, however there still remains little physiological evidence on the role of KIF21A *in vivo*. IHC analysis of an Asian population in this study should therefore be extended to Caucasian cohorts. Furthermore, given many known KIF proteins occur in mice [4], they would be an ideal animal model to knockout genes and explore physiological effects in tumour xenografts. KIF21A's biological pathways are also poorly understood, and could be discovered through microarray analysis of KIF21A-silenced cells. Any upstream or

downstream targets of KIF21A that are known breast cancer oncogenes would provoke interesting follow-up studies, and have the potential to be targeted alongside KIF21A in combinative therapy.

Because of the lack of primary data, KIF21A and many other kinesins are yet to be considered therapeutic targets or prognostic predictors, despite common consensus that kinesins play a vital role in breast carcinogenesis [8]. If mounting evidence supports either a tumour-suppressive or oncogenic role for KIF21A in the metastatic cascade, its pathways could be modulated through targeted chemotherapeutic strategies. Future studies should therefore attempt to uncover the structure of KIF21A's ATP-, microtubule- and enzyme-binding sequences, which could lead to the development of KIF21A inhibitors and enhancers. Identifying binding partners has enormous therapeutic potential, because drugs that mimic those partners could bind to allosteric pockets to either promote or suppress KIF21A and its effects on carcinogenesis.

4.7 Conclusions

In summary, this study illustrates the potential involvement of KIF21A in breast cancer pathogenesis and progression. However, the conflicting outcomes of *in vitro* and *in vivo* data means no definitive conclusions can be drawn about KIF21A's role in breast cancer. This may be due to ethnic differences, the possible multi-functionality of KIF21A, or even study limitations, and warrants further investigation into the influence of KIF21A *in vivo*, the molecules it interacts with, and its potential as a prognostic biomarker. Such knowledge could lead to the development of novel chemotherapeutic strategies to mediate its function and enhance prognostic outcomes.

TABLES

Gene Target	Target Sequence	Concentration Used
KIF21A (Homo sapiens)	5'– CCCUUACAGAAGCCCGAUAtt–3'	10nM
KIF21A(Homo sapiens)	5'– GUAAGACCCAUGUCAGAUAtt– 3'	10nM

Table 1. siRNAs used and their corresponding target sequences. Two different KIF21A siRNA sequences were tested to ensure reliability in results.

Gene	Forward Primer	Reverse Primer	Amplicon Size (bp)
<i>KIF21A</i>	5'–AATGCTGTCAGGATG TGGGA–3'	5'–ACTCACAGTCCCAAGA GCTC–3'	186
<i>β-ACTIN</i>	5'– TGGCACCACACCTTCTAC AAT–3'	5'–GATAGCACAGCCTGGA TAGCA–3'	166
<i>GAPDH</i>	5'– GAAGGTGAAGGTCGGAG TCAACG–3'	5'–TGCCATGGGTGGAATC ATATTGG–3'	157

Table 2. Primer sequences used during qRT-PCR.

Primary antibody	Catalogue number	Dilution	Secondary antibody	Catalogue number	Dilution
KIF21A	Aviva Systems ARP33932_P050	1:1000	Polyclonal goat anti- rabbit HRP	Dako P0448	1:5,000
β -actin	Sigma Aldrich A2228	1:10,000	Anti-mouse IgG HRP	GE Healthcare NA9310	1:15,000

Table 3. List of primary and secondary antibodies used in Western blotting and their dilutions. Detection of bound antibodies was performed using either the Pico or Femto Substrate Systems (Thermo Fisher Scientific). The resulting blots were scanned on X-ray films using a GS-800 Calibrated Imaging Densitometer (Bio-Rad). The optical densities of protein bands were analysed with Quantity One Version 4.1.1 software (Bio-Rad).

Clinicopathological Feature	Number of Cases (%)	Clinicopathological Feature	Number of Cases (%)
<i>Age</i>		<i>Race</i>	
≤ 53 yrs old	149 (56.7)	Chinese	219 (83.3)
> 53 yrs old	114 (43.3)	Non-Chinese	44 (16.7)
<i>Tumour size</i>		<i>Lymphovascular invasion</i>	
≤ 20 mm	90 (34.2)	No	204 (77.6)
> 20 mm	168 (63.9)	Yes	58 (22.1)
Not available	5 (1.9)	Not available	1 (0.4)
<i>Lymph node involvement</i>		<i>Histological grade</i>	
No	99 (37.6)	Grade 1	40 (15.2)
Yes	137 (52.1)	Grade 2	102 (38.8)
Not available	27 (10.3)	Grade 3	114 (43.3)
<i>ER status</i>		Not available	7 (2.7)
Negative	87 (33.1)	<i>PR status</i>	
Positive	172 (65.4)	Negative	126 (47.9)
Not available	4 (1.5)	Positive	133 (50.6)
<i>HER2 status</i>		Not available	4 (1.5)
Negative	158 (60.1)		
Positive	61 (23.2)		
Not available	44 (16.7)		

Table 4. Distribution of Clinicopathological Data in Breast IDC Patients. Abbreviations:

Clinicopathological Parameter		Weighted Average Index (WAI)		Cases with no data	p-value
		≤ 1.0	> 1.0		
		Number of valid cases (%)			
Age	≤ 53 yrs old	89 (59.7)	60 (40.3)	0	0.093
	> 53 yrs old	78 (68.4)	36 (31.6)		
Race	Chinese	141 (64.4)	78 (35.6)	0	0.499
	Non-Chinese	26 (59.1)	18 (40.9)		
Tumour Size	≤ 20 mm	49 (54.4)	41 (45.6)	5	0.030*
	> 20 mm	115 (68.5)	53 (31.5)		
Lymphovascular Invasion	No	128 (62.7)	76 (37.3)	1	0.759
	Yes	38 (65.5)	20 (34.5)		
Lymph Node Involvement	No	63 (63.6)	36 (36.4)	27	0.577
	Yes	93 (67.9)	44 (32.1)		
Histological Grade	Grade 1	19 (47.5)	21 (52.5)	7	0.002**
	Grade 2	63 (61.8)	39 (38.2)		
	Grade 3	84 (73.7)	30 (26.3)		
Oestrogen Receptor	Negative	56 (64.4)	31 (35.6)	4	0.892
	Positive	108 (62.8)	64 (37.2)		
Progesterone Receptor	Negative	83 (65.9)	43 (34.1)	4	0.440
	Positive	81 (60.9)	52 (39.1)		
HER2	Negative	102 (64.6)	56 (35.4)	44	0.166
	Positive	33 (54.1)	28 (45.9)		

ER: oestrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2.

Table 5. Clinicopathological parameters of breast IDC cases correlated against KIF21A expression in epithelial nucleus of tumour cells. Abbreviations: HER2: human epidermal growth factor receptor 2. * $p < 0.05$, ** $p < 0.01$ (Fisher's exact test for nominal parameters, Kendall's tau-c test for ordinal parameters).

Timeline	KIF21A WAI	Total	No. of events	Censored		Log-rank p (Mantel-Cox)
				No.	Percent	
Overall Survival	≤ 1.0	166	43	123	74.1%	0.012*
	>1.0	96	12	84	87.5%	
	Overall	262	55	207	79.0%	
Survival After Recurrence	≤ 1.0	67	43	25	37.3%	0.025*
	>1.0	31	12	21	67.7%	
	Overall	98	55	46	46.9%	
Disease-free Survival	≤ 1.0	165	67	98	59.4%	0.187
	>1.0	95	31	64	67.4%	
	Overall	260	98	162	62.3%	

Table 6. Data summary of nuclear KIF21A epithelial staining, cause-specific mortality (OS and SAR) and recurrence (DFS) in breast IDC patients over 156-month follow-up period. An event is defined as either cause-specific death (for OS and SAR timelines) or

Timeline	Parameter	Reference Group ^a (HR = 1)	Univariate Analysis ^c			Multivariate Analyses					
						Model A ^d			Model B ^e		
			HR	95% CI	p value	HR	95% CI	p value	HR	95% CI	p value
Overall survival	Tumour Size	≤ 20 mm	2.91	1.42 – 5.96	0.002**	2.55	1.24 – 5.25	0.011*	2.20	0.88 – 5.50	0.090
	Histological Grade	Low ^b	1.67	1.10 – 2.54	0.012*	1.42	0.91 – 2.21	0.121	0.99	0.57 – 1.72	0.968
	KIF21A WAI	Low	0.45	0.24 – 0.85	0.012*	0.39	0.19 – 0.81	0.012*	0.34	0.14 – 0.82	0.017*
Survival after recurrence	Tumour Size	≤ 20 mm	2.24	1.09 – 4.590	0.023*	2.03	0.99 – 4.19	0.055	1.30	0.49 – 3.45	0.604
	KIF21A WAI	Low	0.49	0.26 – 0.93	0.025*	0.52	0.27 – 1.01	0.049*	0.37	0.13 – 1.04	0.048*
Disease-free survival	Tumour Size	≤ 20 mm	1.59	1.01 – 2.50	0.042*	1.21	0.72 – 2.03	0.477	1.29	0.77 – 2.14	0.335
	Lymph Node Involvement	No	1.83	1.16 – 2.88	0.008**	1.44	1.18 – 1.75	0.001**	1.87	1.17 – 2.98	0.009**
	Histological Grade	Low ^b	1.53	1.13 – 2.07	0.021*	1.22	0.86 – 1.74	0.261	1.30	0.92 – 1.83	0.142
	ER	Negative	0.50	0.33 – 0.74	0.001**	0.47	0.30 – 0.73	0.001**	0.56	0.29 – 1.07	0.001**
	KIF21A WAI	Low	0.75	0.49 – 1.15	0.187	-	-	-	0.47	0.30 – 0.73	0.080

recurrence (DFS timeline), while censored cases represent patients who either did not experience an event during the entire follow-up period, or who withdrew from the study during the follow-up period without experiencing an event. * *Log-rank* $p < 0.05$.

Table 7. Univariate (Kaplan-Meier) and multivariate (Cox proportional hazards regression) analysis of cause-specific survival in breast IDC patients. Abbreviations: HR: hazard ratio; CI: confidence interval; ER: oestrogen receptor. ^a Reference groups (HR = 1) were used as the denominator in hazard ratio calculations; ^b Histological grades 1 and 2 were binned into “low” grade, while “high” is classified as grade 3; ^c Kaplan-Meier univariate analysis performed using the log-rank (Mantel-Cox) test; ^d Model A: Cox proportional hazards model including only significant ($p < 0.05$) variables identified from the Kaplan-Meier univariate analysis; ^e Model B: Cox proportional hazards model including all variables. * $p < 0.05$, ** $p < 0.01$.

FIGURE LEGENDS

Figure 1. KIF21A silencing efficiencies. A,B) qRT-PCR analysis of KIF21A silencing using two siRNA sequences, siKIF21A-1 and siKIF21A-2, in MCF-7 (A) and MDA-MB-231 (B) cells showed a reduction in KIF21A mRNA expression upon silencing. C,D) Western blot protein band densitometry showed a significant reduction in the optical density of KIF21A protein bands in silenced cells for both cell lines. All protein densities from silenced cells were normalised against β -actin. For all figures: scrambled siRNA was used as the negative control. Values are mean $\pm$ SEM. n = 3 for each group, * p < 0.05, ** p < 0.01, *** p < 0.001 (One-way ANOVA with Tukey's multiple comparisons post-hoc test).

Figure 2. Transwell migration assays of MCF-7 and MDA-MB-231 cells following KIF21A silencing. (A,C) There was a significant decrease in migrated MCF-7 cells and MDA-MB-231 cells following KIF21A silencing for both silenced groups. (B,D) Representative photomicrographs (10x magnification) of cell migration in MCF-7 cells and MDA-MB-231 cells. For all figures: values are mean $\pm$ SEM, n = 3 for each group, ** p < 0.01, *** p < 0.001, **** p < 0.0001 (One-way ANOVA with Tukey's multiple comparisons post-hoc test). Scale bars represent 100 μ m.

Figure 3. Matrigel invasion assays of MCF-7 and MDA-MB-231 cells following KIF21A silencing. (A,C) There was a significant decrease in invasive MCF-7 cells and MDA-MB-231 cells following KIF21A silencing for both silenced groups. (B,D) Representative photomicrographs (10x magnification) of cell invasion in MCF-7 cells and MDA-MB-231 cells. For all figures: values are mean $\pm$ SEM, n = 3 for each group, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 (One-way ANOVA with Tukey's multiple comparisons post-hoc test). Scale bars represent 100 μ m.

Figure 4. Cell proliferation and adhesion assays in MCF-7 and MDA-MB-231 cells following KIF21A silencing. (A,B) Serum-starved proliferation assay of MCF-7 and MDA-MB-231 cells following KIF21A silencing. There were no significant differences observed in cell proliferation following KIF21A silencing for both cell lines. (C,D,E,F) Adhesion assays of MCF-7 and MDA-MB-231 cells following KIF21A silencing. For all figures: absorbance (of formazan) was measured at 490 nm and indicates the relative percentage of live cells;

values are mean $\pm$ SEM; n = 3 (proliferation assays) or 7 (adhesion assays) for each group, * p < 0.05 (One-way ANOVA with Tukey's multiple comparisons post-hoc test).

Figure 5. (A,B,C) Representative photomicrographs of KIF21A immunoreactivity in breast IDC tissue. Staining was predominately localised to the epithelial nucleus of (A) tumour cells and (B) adjacent normal tissue, while cytoplasmic staining was rare. (C) Negative control slide. Scale bars represent 50 μ m. All photomicrographs are at 40x magnification. (D) There were no significant differences in KIF21A protein expression between breast IDC tumour tissues and adjacent normal tissues, as measured by IHC. Values are mean WAI $\pm$ SEM. n = 287 (263 tumour, 24 normal) (Mann-Whitney test). Abbreviations: WAI: Weighted Average Intensity.

Figure 6. Kaplan-Meier curves for overall cause-specific survival rate (A), cause-specific survival rate after recurrence (B), and disease-free survival (C). KIF21A protein levels in the epithelial nuclei of tumour cells showed prognostic roles in survival (A,B), but not recurrence (C). Patients with low KIF21A expression in breast cancer tissue had significantly shorter survival than those with high KIF21A expression.

FIGURES

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

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