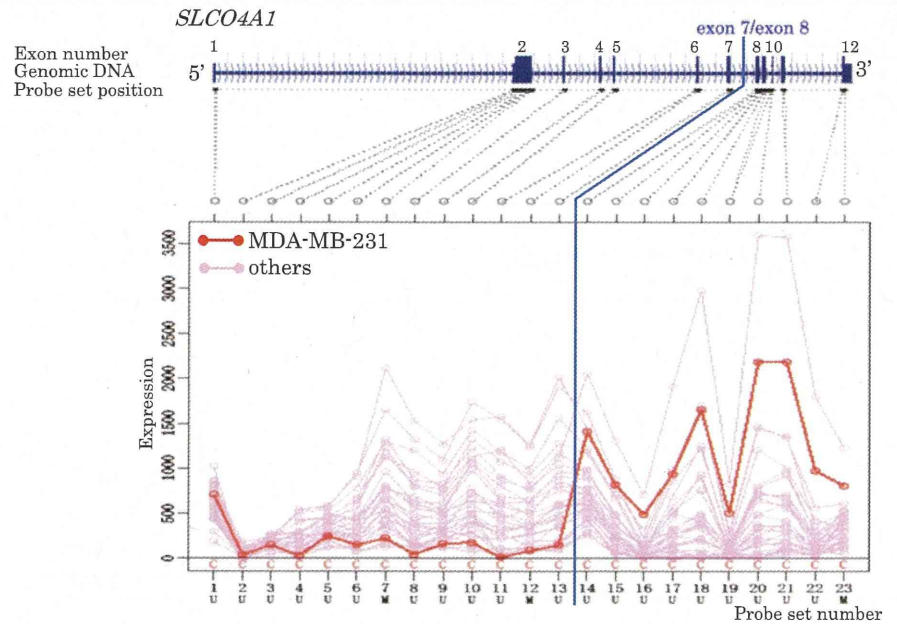

Figure 7 Exon expression profiles of candidate gene SLCO4A1 in MDA-MB-231 cell.

standardized value was used as an index of the expression level of each probe set. The probe sets were then separated in a transcript cluster into 5′ and 3′ terminal groups by one arbitrary point, and the expression level change was monitored between groups by t-test.

Comparing the proposed methodology with Lin's method, a common feature is that signal intensity is normalized based on the relative relation to reference samples, aiming to compare the expression levels of all probe sets in a gene. The most important difference is

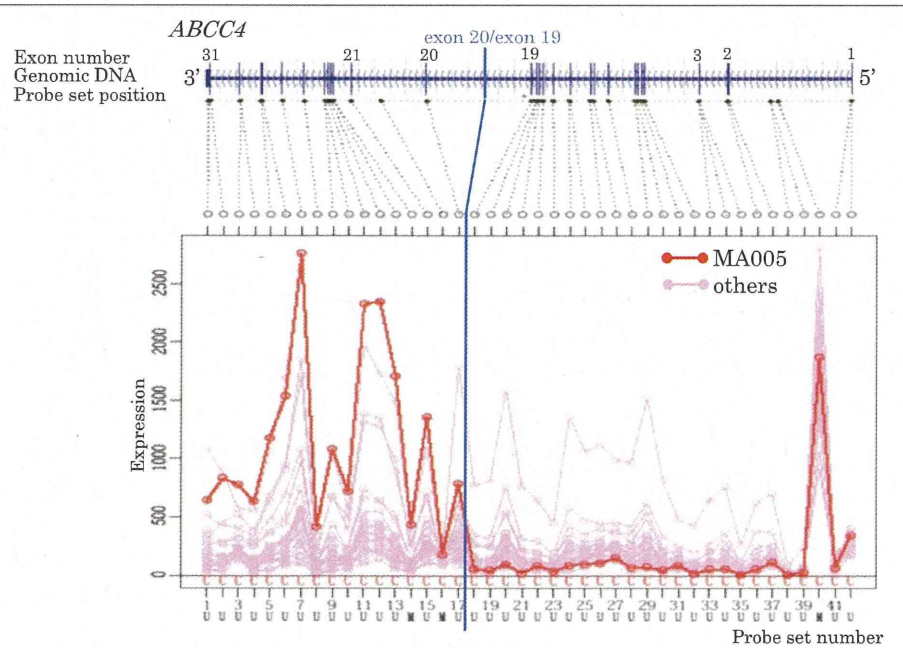

Figure 8 Exon expression profiles of candidate gene ABCC4 in MA005 cell.

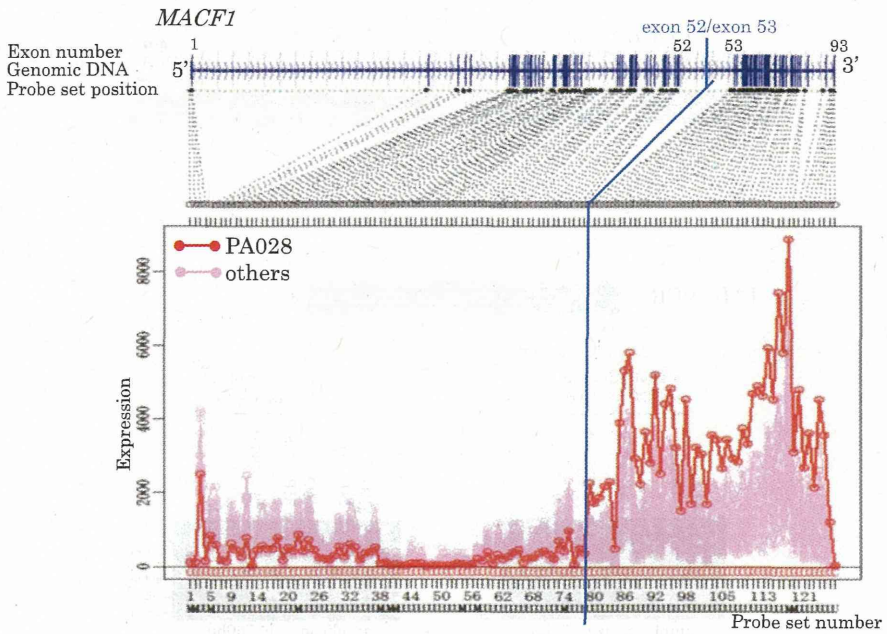

Figure 9 Exon expression profiles of candidate gene MACF1 in PA028 cell.

the strategy of normalizing. In Lin's method, it is thought that normalized values have a fixed quantity, which is an advantage to evaluate whether the magnitude of the change is significant; however, this is influenced easily by outlier intensities, which are generated frequently in microarray experiments. On the other hand, in the developed program, the magnitude of the change is not evaluated appropriately, but it has the advantage that the result is not influenced easily by the outlier value because the expression intensity is converted into the rank.

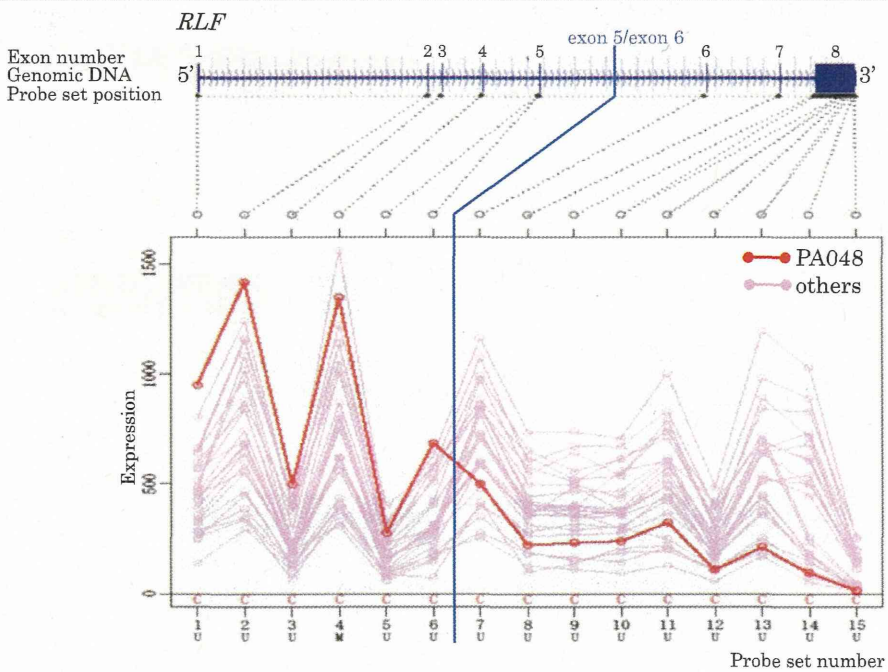

Figure 10 Exon expression profiles of candidate gene RLF in PA043 cell.

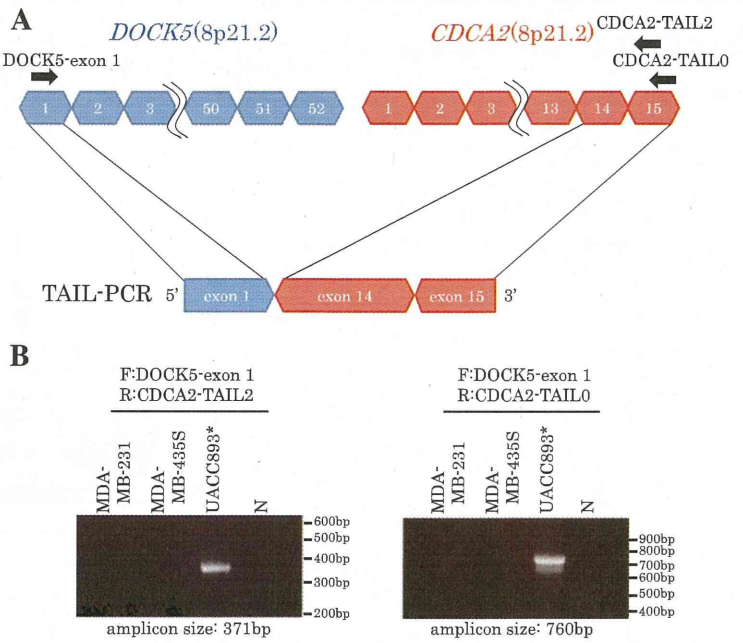

Figure 11 TAIL-PCR detection and RT-PCR confirmation of fusion gene DOCK5-CDCA2. Acquired fusion fragments by TAIL-PCR and exon structures of fusion partners are shown in **(A)** Red blocks are exons of candidate gene, blue blocks are exons of detected genes by TAIL-PCR. Arrows are primers for RT-PCR. RT-PCR confirmations for indicated samples are shown in **(B)** F: forward primer, R: reverse primer, *: detected samples in the program.

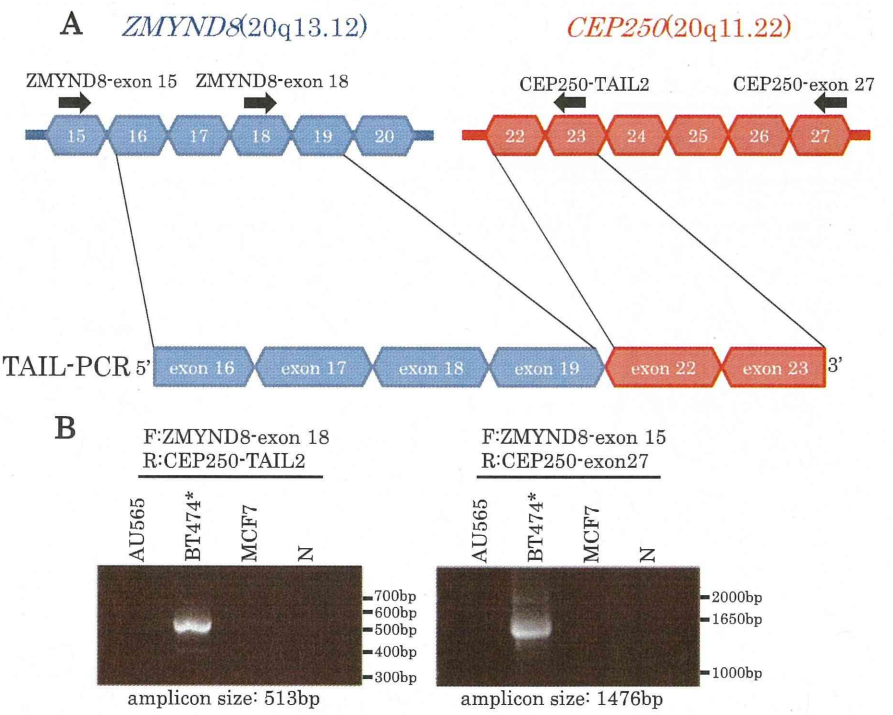

Figure 12 TAIL-PCR detection and RT-PCR confirmation of fusion gene ZMYND8- CEP250. Acquired fusion fragments by TAIL-PCR and exon structures of fusion partners are shown in **(A)** and **(B)** like Figure 11.

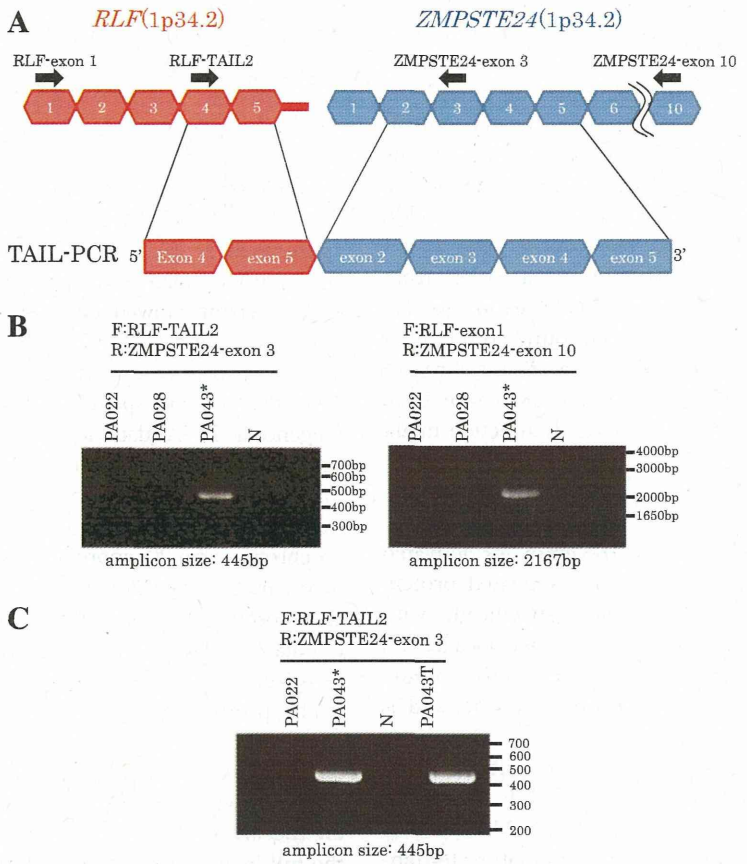

Figure 13 TAIL-PCR detection and RT-PCR confirmation of fusion gene RLFZMPSTE24. Acquired fusion fragments by TAIL-PCR and exon structures of fusion partners are shown in **(A)** and **(B)** like Figure 11, and RT-PCR detections for pancreatic cancer clinical tissue PA043T is shown in **(C)**.

Points to be improved and limitations

The analysis result would possibly change depending on the selection of reference samples, because signal intensities are converted into relative values by comparing with other samples. Lin's method has the same problem. It is thought that ideal reference samples for the program would show moderate variance of the gene expression level. Although cancer cell lines and healthy cells from the same organ were used in this research, further examination is necessary to assess whether this is the best choice. In addition, parameter optimization (degree of rank change, standard deviation and so on) for the reference samples is required.

The following points are limitations of this method, and alternative methods are needed. As this method detects the intragenic expression change in fusion partner genes, the method cannot detect the genes with no significant expression change between exons. Additionally, breakpoint detection from exon array data depends on the genomic position of the probe set. Thus, this method is not able to identify breakpoints on genomic DNA in detail.

Contribution of the fusion genes to cancer

The discovery of fusion genes that contribute to the pathology (tumorigenesis, metastasis etc.) are hoped from the viewpoint of the diagnosis and treatment of cancer. Considering the functional aspect of the fusion gene, it is important to incorporate other information, such as protein domain composition, when prioritizing novel, biologically relevant genomic aberrations [25].

Although three novel fusion genes were identified in this research, their function and contribution to cancer are unclear.

DOCK5-CDCA2:

DOCK5 (dedicator of cytokinesis 5) is a member of the DOCK family of guanine nucleotide exchange factors which function as activators of small G proteins [26]. Although DOCK5 is predicted to activate the small G

protein Rho and Rac, its function and signaling properties are poorly understood. CDCA2 (cell division cycle associated 2) recruits protein phosphatase 1 to mitotic chromatin at anaphase and into the following interphase, regulating the chromosome structure during mitosis [27]. Because *DOCK5* and *CDCA2* show out-of-frame fusion, it is thought that the amino acid sequence of *CDCA2* is disrupted and a premature termination codon appears in *CDCA2* exon 14. The fusion gene might therefore produce a short protein, 42aa (14aa from *DOCK5* exon 1, and 28aa from *CDCA2* exon 14). No functional protein domains have been found so the function of the fusion protein is unclear. Significant chromosome loss and underexpression of *DOCK5* have been reported in osteosarcoma [28]. DOCK5 dysfunction might contribute to tumors.

ZMYND8-CEP250:

ZMYND8 is a member of RACK (receptor for activated C-kinase) family proteins that anchor activated protein kinase C (PKC). ZMYND8 interacts specifically with PKCβI and is predicted to regulate subcellular localization and activity [29]. In addition, ZMYND8 contains a bromo domain, a PWWP domain, and two zinc fingers, and is thought to be a transcriptional regulator. CEP250 is a core centrosomal protein required for centriole-centriole cohesion during interphase of the cell cycle [30], but details of the mechanism are not well known. *ZMYND8-CEP250* is also an out-of-frame fusion gene, so a premature termination codon appears in *CEP250* exon 24 and is likely to express a 1121aa protein (994aa from *ZMYND8* exon 1–19, and 127aa from *CEP250* exon 22–24). The down-regulation of PKCβ1 protein expression has been reported in colon cancer [31]. The PKCβ1 binding site in the C terminal region of ZMYND8 racks in the predicted fusion protein. Formation of the fusion gene may lead to the low activity of PKCB1, and may contribute to cancer, or deregulation of the transcript regulatory network managed by ZMYND8 might cause cancer.

RLF-ZMPSTE24:

RLF is predicted as a transcription factor with zinc fingers from the amino acid sequence. It is reported that RLF forms a fusion gene with the LMYC gene in lung cancer [32]. The fusion gene RLF-LMYC contributes to carcinogenesis by changing the LMYC manifestation of a gene [33]. ZMPSTE24 performs a critical endoproteolytic cleavage step to generate mature lamin A, a major component of the nuclear lamina and nuclear skeleton [34]. Lack of functional ZMPSTE24 results in progeroid phenotypes, including genomic instability in mice and humans [35,36]. *RLF-ZMPSTE24* is an in-frame fusion gene, which may expresses the 704aa protein (270aa from *RLF* exon 1–5, and 434aa from *ZMPSTE24* exon 2–10). The known function domains of *RLF* are not contained in the fusion gene, and no change of *ZMPSTE24* expression level is observed in Exon Array data. Functional change of ZMPSTE24 may induce DNA damage and lead to cancer.

Genomic structure of the fusion genes

RLF and *ZMPSTE24* genes located on chromosome 1, approximately 20 kb apart, have the same orientation. Southern blot analysis with a probe hybridizing to *RLF* intron 5 region showed chromosome rearrangement (data not shown), and a fragment that is part of *RLF* intron 5 fused to a part of *ZMPSTE24* intron 1 was obtained by TAIL-PCR for the upstream region of *ZMPSTE24* exon 2 on genomic DNA (data not shown). Both parts fused in the opposite orientation; therefore, the cause of the gene fusion, *RLF-ZMPSTE24*, might be chromosome inversion with some deletion. *ZMYND8* and *CEP250* genes were located on chromosome 20, approximately 12 Mb apart, in opposite orientation. *DOCK5* and *CDCA2* genes were located on chromosome 8, approximately 50Kb apart, in the same orientation. The mechanisms of gene fusions remain to be revealed.

The proposed method might be applied to not only Exon Array but also the Affymetrx GeneChip Gene 1.0 ST Array (Gene Array) with some improvements. Gene Array, in which each of the 28,869 genes is represented on the array by approximately 26 probes spread along the full length of the gene, is widely used for global gene expression analysis. Using this method for more samples, it is thought that fusion genes can be identified. This is expected to lead to new diagnostic methods and treatment strategies.

Additional files

Additional file 1: Selected genes by the program in 24 breast cancer cell lines.

Additional file 2: Selected genes by the program in 20 pancreatic cell lines.

Competing interests

The authors declare that they have no competing financial interests or other conflicts of interest.

Authors' contributions

YW carried out sample preparation, Exon Array Experiments, TAIL-PCR, manuscript writing and helped to develop the algorithms. MM developed the majority of algorithms, helped to draft the manuscript. MS contributed cell cultue and TAIL-PCR, helped to draft the manuscript. MU and SM contributed statistical support and data processing. KN contributed preparation of clinical samples. TN and YM contributed to study conception, and critical manuscript review. All authors read and approved the final manuscript.

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Cancer Research

BRCA2 Phosphorylated by PLK1 Moves to the Midbody to Regulate Cytokinesis Mediated by Nonmuscle Myosin IIC

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Tumor and Stem Cell Biology

Cancer Research

BRCA2 Phosphorylated by PLK1 Moves to the Midbody to Regulate Cytokinesis Mediated by Nonmuscle Myosin IIC

Miho Takaoka[1], Hiroko Saito[2], Katsuya Takenaka[1], Yoshio Miki[1,2], and Akira Nakanishi[1]

Abstract

Cytokinesis is the critical final step in cell division. BRCA2 disruption during cytokinesis is associated with chromosome instability, but mechanistic information is lacking that could be used to prevent cancer cell division. In this study, we report that BRCA2 phosphorylation by the mitotic polo-like kinase (PLK1) governs the localization of BRCA2 to the Flemming body at the central midbody, permitting an interaction with nonmuscle myosin IIC (NM-IIC). Formation of an NM-IIC ring-like structure at the Flemming body shows that the IIC-ring relies on its ATPase activity stimulated by interaction with BRCA2 and associated proteins. Notably, inhibiting this binding inactivated the ATPase activity, causing disassembly of the IIC-ring, defective formation of the midbody, and interruption of cytokinesis. An analysis of cancer-associated mutations in BRCA2 at the PLK1-binding site suggests that they may contribute to cytokinetic defects by altering BRCA2 localization. Our findings suggest that BRCA2-dependent IIC-ring formation is a critical step in proper formation of the midbody, offering an explanation for how chromosome instability may arise in breast cancer. *Cancer Res; 74(5); 1518–28. ©2014 AACR.*

Introduction

Germline mutations in the *BRCA2* gene have been reported to increase the risk of developing breast and ovarian cancer. The BRCA2 protein has multiple functions, including DNA double-strand break repair (1–3), and the regulation of centrosome amplification and localization (4, 5). BRCA2 also contributes to the regulation of cytokinesis (6, 7). During anaphase, constriction of the actomyosin ring leads to formation of a cleavage furrow (8–10). Continued furrowing results in the formation of a narrow intercellular bridge, which contains the midbody, consisting of a bundled microtubule and a ring-like structure called the Flemming body within its central portion (11). Several studies have implicated various protein factors in the modulation of cytokinesis by BRCA2 (12–14). Disruption of BRCA2 during cytokinesis leads to disorganization of myosin-II at the cleavage furrow and the intercellular bridge (6).

Mitotic polo-like kinase 1 (Plk1) is a key regulator of mitosis from mitotic initiation to cytokinesis. Plk1 contains a serine/threonine kinase domain followed by the carboxy-terminal polo-box domain (PBD), which binds to phosphopeptides within a consensus motif of S-[pS/pT]-[P/X] (15, 16). The PBD regulates cellular function, the interaction with substrates, and the subcellular localization of Plk1 (17). Plk1 is also required for appropriate localization of substrates (18). Previous studies revealed that Plk1 binds to the N-terminal region of BRCA2 and phosphorylates Ser193, and that this phosphorylation is enhanced as mitosis progresses (19, 20). However, the specific Plk1-binding site within this region of BRCA2 has not been identified. The role of BRCA2 phosphorylation in the maintenance of genome stability also remains unclear.

Nonmuscle myosin-II (NM-II) proteins in humans are hexamers, consisting of a pair of heavy chains and two pairs of light chains that hydrolyze MgATP. They are members of a family of actin-binding motor proteins that play essential roles in cellular processes such as cell division and embryonic development. The NM-II family comprises three isoforms: NM-IIA, NM-IIB, and NM-IIC. These contain different nonmuscle myosin heavy chains (NMHC-IIA, NMHC-IIB, and NMHC-IIC) that are encoded by *MYH* (myosin heavy chain) *9*, *MYH10*, and *MYH14*, respectively (21, 22). The N-terminal region of the NMHC-II protein consists of a globular head containing the actin-binding region, an ATPase domain, and a Src homology 3 (SH3)-like domain (myosin head; refs. 23, 24). NM-IIC is alternatively spliced both in loop-1 and loop-2. Isoform NM-IIC0 contains no inserts in either of the loops. An 8–amino acid extension in the loop-1 region is present in isoforms NM-IIC1 and NM-IIC1C2. Isoform NM-IIC1C2 displays a 33–amino acid extension in the loop-2 region. The presence of 8 amino acid insert in NM-IIC increases the actin-activated ATPase activity (25). The C-terminal deletion isoform of NM-IIC is already present in the Ensembl database (http://www.ensembl.org/index.html; MYH14-007, Protein ID: ENSP00000469573). Jana

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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and colleagues reported the localization of NM-IIC to the midbody during the process of abscission. In that report, they confirmed that NM-IIC1 is required for cytokinesis (25). Accordingly, we made use of this isoform. In this study, we suggest that BRCA2-dependent IIC-ring formation represents a key step in proper midbody formation, and that a hereditary breast cancer-associated mutation within the Plk1 interaction motif of BRCA2 affects the localization of BRCA2 to the Flemming body, resulting in cytokinesis defects.

Materials and Methods

Detailed descriptions of plasmids, transfections, siRNA treatment, antibodies, immunofluorescence and three-dimensional (3D) reconstitutions, immunoprecipitation, Western blot analysis, binding assays, glycerol density gradient centrifugation, time-lapse microscopy, measurement of ATPase activity, cell-cycle analysis, and statistical analysis are provided in the Supplementary Materials and Methods.

Cell culture

HeLaS3 and COS-7 cells were purchased from the RIKEN GENBANK, and U2OS and A549 were from American Type Culture Collection (ATCC). These cells were cultured in Dulbecco's Modified Eagle Medium containing 10% fetal calf serum (FCS). MCF7 was purchased from ATCC and cultured in Eagle's Minimum Essential Medium containing 10% FCS, 0.01 mg/mL bovine insulin, and 1% sodium pyruvate. The identities of HeLaS3, U2OS, A549, and MCF7 cell lines were confirmed by short tandem repeat (STR) profiling by BEX CO., Ltd and used within 6 months of testing. COS-7 cells (monkey cell line) were not analyzed by STR profiling because STR profiling is method for authentication of human cell lines.

Midbody isolation

Mitotic cells (A549) were released from colcemid treatment (50 ng/mL) for 17 hours by washing twice with fresh medium. After incubating at 37°C for 60 minutes, the cells were collected. Midbody isolation was performed according to the methods of Mullins and McIntosh (26).

Measurement of ATPase activity

COS-7 cells were transfected with plasmids expressing HA-tagged NMHC-IIC and FLAG-tagged BRCA2. After 24 hours, cells (5×10^6) were lysed in lysis buffer [20 mmol/L Tris-HCl (pH 8.0), 100 mmol/L NaCl, 1 mmol/L EDTA, 1% NP-40, and protease inhibitors]. BRCA2 and NMHC-IIC were immunoprecipitated by anti-FLAG or anti-HA antibodies, respectively. Each sample was eluted by 40 μL elution buffer (50 mmol/L glycine, pH 2.8), which was immediately neutralized with 1 mol/L Tris. BRCA2-FLAG and NMHC-IIC-HA were mixed and incubated at room temperature for 1 minute and then placed into a solution for measurement of ATPase activity. F-actin was added immediately before the measurement. ADP production during ATP hydrolysis was measured as a change in NADH concentration. NADH absorbance was measured every 2 seconds at 340 nm and the rate of NADH consumption was interpreted as the ATPase activity.

Results

Localization of BRCA2 to the Flemming body through phosphorylation by Plk1

To analyze the role of phosphorylation of BRCA2 Ser193 by Plk1, we generated an antibody (193pBRCA2) specific for this phosphoprotein and confirmed the specificity of the 193pBRCA2 antibody by performing a competition assay using pS^{193}-Peptides and treatment with protein phosphatase (PP2A; Supplementary Fig. S1A and S1B). This antibody recognized Ser^{193}-phosphorylated BRCA2 (pS^{193}-BRCA2) in anti-BRCA2 (Ab-1) immunoprecipitates from midbody lysates (Supplementary Fig. S1C).We observed its localization during the cell cycle by immunofluorescence microscopy. BRCA2 localized to the midbody (particularly at the Flemming body) during cytokinesis of mitotic HeLaS3 cells (Fig. 1A). Similar findings were observed in A549, MCF7, and U2OS cells (Supplementary Fig. S1D). The phosphorylated BRCA2 also localized to the Flemming body (Fig. 1A). In contrast, BRCA1 did not localize to the Flemming body (Fig. 1A and Supplementary Fig. S1E). This result is consistent with that of a previous study by another group (13). We then isolated the midbodies from HeLaS3 cells (Supplementary Fig. S1F) and compared the extent of BRCA2 phosphorylation in midbody lysates with that in whole-cell lysates (Fig. 1B). A greater fraction of the BRCA2 was phosphorylated in the midbody. Using immunofluorescence microscopy, we showed that Plk1 colocalizes with pS^{193}-BRCA2 at the Flemming body (Fig. 1C). Assays of COS-7 cells transfected with BRCA2-FLAG and Plk1-HA demonstrated that BRCA2 and Plk1 coimmunoprecipitated (Fig. 1D). We located a Plk1-binding motif (S-T-P sequence at codons 76–78) in the N-terminal region of BRCA2. A Thr77-phosphorylated peptide corresponding to this region (a.a. 73–82 of BRCA2) was synthesized for *in vitro* binding analyses. Streptavidin bead–immobilized p-Thr77 peptide precipitated Plk1 from mitotic HeLaS3 cell lysates, whereas unphosphorylated peptide or peptide-free streptavidin beads did not (Fig. 1E). Plk1 was not precipitated from S-phase lysates due to low expression.

This Plk1-binding site is conserved among diverse species (Supplementary Fig. S1G). A hereditary breast cancer-associated missense mutation (T77A) within this site is annotated in the Breast Cancer Information Core (BIC) database (http://research.nhgri.nih.gov/bic/). We constructed plasmids encoding a truncated N-terminal region [BRCA2 (R1)-FLAG (WT):1–157 a.a.] containing either the wild-type Plk1-binding site or this mutant, BRCA2 (R1)-FLAG (T77A; Supplementary Fig. S1H). The mutant protein was unable to interact with endogenous Plk1 in HeLaS3 cells (Supplementary Fig. S1I), indicating that this mutation disrupted the interaction with Plk1. Next, we examined whether overexpression of this truncated region could compete with endogenous BRCA2. Coimmunoprecipitation assays were performed in HeLaS3 cells expressing BRCA2 (R1)-FLAG (WT). It was demonstrated that this region inhibited the binding of endogenous BRCA2 to Plk1 (Supplementary Fig. S1J). Also, the endogenous BRCA2 ceased to be detected at the Flemming body following ectopic expression of this region (Supplementary Fig. S1K). These results suggested a dominant-negative effect of HA-BRCA2 (R1) upon endogenous BRCA2. A distinct HA-tagged BRCA2 region [HA-BRCA2 (R2):