Functional differences between wild-type and mutant-type BRCA1-associated protein 1 tumor suppressor against malignant mesothelioma cells

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Malignant mesothelioma (MM) is an aggressive neoplasm which was primarily associated with widespread use of asbestos. It is usually resistant to conventional multimodal therapies including chemotherapy, radiotherapy, and surgical therapy. The prognosis of patients with MM remains very poor; the median survival of MM patients after diagnosis is only 7–12 months.

Previous studies have identified frequent genetic alterations of the two tumor suppressor genes, CDKN2A and NF2, in MM. NF2 encodes merlin, an upstream regulator of the Hippo signaling pathway, and we recently reported that BAP1 3’ side resulted in both inhibition of cell proliferation and anchorage-independent cell growth, whereas BAP1 mutants of a missense or C-terminal truncated form showed impaired growth inhibitory effects. Next, we studied how BAP1 is involved in MM cell survival after irradiation (IR), which causes DNA damage. After IR, we found that both WT and mutant BAP1 were similarly phosphorylated and phospho-BAP1 localized mainly in the nucleus. Interestingly, BRCA1 proteins were decreased in the MM cells with BAP1 deletion, and transduction of the mutants as well as WT BAP1 increased BRCA1 proteins, suggesting that BAP1 may promote DNA repair partly through stabilizing BRCA1. Furthermore, using the MM cells with BAP1 deletion, we found that WT BAP1, and even a missense mutant, conferred a higher survival rate after IR compared to the control vector. Our results suggested that, whereas WT BAP1 suppresses MM cell proliferation and restores cell survival after IR damage, some mutant BAP1 may also moderately retain these functions.

BAP1 is a nuclear-localized deubiquitinating (DUB) enzyme with an NH2-terminal ubiquitin COOH-terminal hydrolase (UCH) domain and a COOH-terminal domain which contains two nuclear localization signals (NLS). BAP1 has been suggested to act as a tumor suppressor with possibly three functions. First, BAP1 acts as a transcriptional factor, associating with host cell factor 1, YY1, and E2F1. The complex of these factors is recruited to various promoters to upregulate gene expression. Second, BAP1 acts as a component of the Polycomb repressive deubiquitinase (PR-DUB) complex, associating with ASXL1. The PR-DUB complex deubiquitinates the histone H2A (H2AK119ub1), leading to gene repression. Third, BAP1 contributes to the process of DNA repair.

The possible function BAP1 may have in the DNA repair process has not yet been made clear. The homologous recombination (HR) pathway, one of the major pathways of DNA repair, includes many proteins, some of which may be potential substrates for BAP1-mediated ubiquitin hydrolysis. Eletr et al. reported that BAP1 is phosphorylated at serine 592 following DNA damage, that phospho-BAP1 causes deubiquitination of H2AK119ub1, and that this signal promotes the HR pathway. Yu et al. also found six irradiation (IR)-induced phosphorylation sites in BAP1 and showed that mutation of...
these residues inhibits the recruitment of BAP1 to the double-strand break (DSB) sites. Furthermore, Ismail et al. suggested that BAP1 could function for HR repair in the BRCA1-related pathway, and they hypothesized that BAP1 enables BRCA1 to readily accumulate DSB sites. These three reports suggested that the DUB catalytic activity of BAP1 is critical for promoting DNA repair.

In this study, we investigated the BAPI gene status using MM cell lines, most of which were established from Japanese MM patients. We further examined the functional differences between WT and mutant forms of BAP1 regarding subcellular localization, cell growth regulation, and the response to IR-induced cellular damage. We found that some mutant forms of BAP1 still retain partial activities of these functions.

Materials and Methods

Cell lines. Nineteen Japanese MM cell lines, namely ACC-MESO-1, -4, Y-MESO-8D, -9, -12, -14, -21, -22, -25, -26B, -27, -28, -29, -30, -45, -48, -61, -72, and -76, were established in our laboratory as reported previously and described elsewhere, and the cells at 10–15 passages were used for assays. For MM cell lines including NCI-H28, NCI-H2052, NCI-H2452, and one immortalized mesothelial cell line, MeT-5A, were purchased from ATCC (Rockville, MD, USA), and cells at 3–5 passages were used. NCI-H290 and NCI-H2452 were the kind gifts of Dr. Adi F. Gazdar (Hamon Center for Therapeutic Oncology, University of Texas Southwestern Medical Center, Dallas, TX, USA). All MM cell lines and MeT-5A were cultured as described in Data S1. Malignant mesothelioma tissue samples from patients for the establishment of cell culture were obtained according to the Institutional Review Board’s approved protocol and with written informed consent from each patient.

Antibodies. For Western blot and immunofluorescence analyses, mouse anti-BAP1 antibody (sc-28383) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and rabbit anti-phospho-BAP1 (Ser592; #9010), rabbit anti-BAP1 (Ser1524; #9009), rabbit anti-phospho-BRCA1 (Ser2681; #9010), rabbit anti-phospho-BRCA1 (Ser1524; #9009), rabbit anti-Lamin-B1 (#12586), and rabbit anti-α-tubulin (#2125) antibodies were from Cell Signaling Technology (Danvers, MA, USA), and mouse anti-α-actin (clone AC74) was from Sigma (St. Louis, MO, USA).

Construction of expression vectors. cDNA fragments of WT or mutant BAPI were amplified by PCR using PrimeSTAR Max DNA polymerase (Takara Bio, Otsu, Japan), and introduced into the pcDNA3.1 V5-His expression vector (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), thereby fusing these cDNAs with the V5-His sequence. The sequences of all constructs were confirmed. To generate BAPI expressing lentiviral vector, cDNA coding for the human BAPI tagged with V5-His was amplified by PCR and cloned into the pLlL3.7 lentiviral vector with an infusion cloning system (Clontech, Mountain View, CA, USA).

Results

BAPI mutations in MM. We carried out mutational analyses of BAPI using 25 MM cell lines. Among 19 cell lines established from Japanese patients, four non-synonymous or insertion/deletion mutations were found: a nonsense mutation in ACC-MESO-4, two truncating mutations in Y-MESO-9 and Y-MESO-14, and a 40-bp insertion mutation at the intron 7–exon 8 junction in Y-MESO-61 (Fig. 1a,b). We also carried out sequence analysis of cDNA synthesized from these cell line RNAs and confirmed the expression of the mutant RNAs (data not shown). As our previous study using array comparative genomic hybridization analysis detected a possible homozygous deletion (HD) in Y-MESO-25 (Fig. S1a), we carried out a genomic PCR analysis and confirmed that this cell line has HD of BAP1 at exons 13–17 (Fig. 1c). In total, we found BAPI mutations in 26% (5/19) of the Japanese MM cell lines (Table 1). In addition, of six cell lines, the mutation status of which has been previously reported by another group, we confirmed the same mutations in two cell lines (NCI-H28 and NCI-H2452) and in the WT among four cell lines (Table 1).

Next, we used Western blot analysis to confirm the inactivation status of BAP1 with the antibody that detects the COOH-terminus of BAP1. Among seven cell lines with a BAP1 mutation, four (NCI-H28, Y-MESO-14, Y-MESO-25, and Y-MESO-61) expressed no band, two (NCI-H2452 and ACC-MESO-4) expressed a very weak band, and one (Y-MESO-9) expressed a short-sized band of BAP1 protein (Fig. 1d). Thus, these aberrant protein expression patterns were consistent with the mutation status.

We compared the mutation status of BAPI with other genes previously reported (Fig. 1e). However, we found no significant associations between BAPI, CDKN2A, NF2, LATS2, and SAV1 mutations, which seem to be in agreement with other published reports. (13)

Effects of BAPI mutation on its own nuclear localization. BAPI contains two NLS at the COOH-terminus, and is primarily localized in the nucleus (17). We used immunofluorescence analysis to determine whether or not the BAPI mutations have an effect on its subcellular localization, because four mutations were predicted to result in the loss of the both NLS and one in the NLS2 at the COOH-terminus among the seven mutations. As expected, NCI-H290 cells, which harbor WT BAPI, showed endogenous BAPI expression primarily in the nucleus (Fig. 2a). In contrast, Y-MESO-9 cells, which lack the NLS2, showed endogenous BAPI mainly in the cytoplasm. These results suggested that nuclear localization of endogenous BAPI is impaired primarily by the loss of NLS, which was consistent with a previous study. (30)

To confirm whether these mutations were the main cause of the aberrant cellular localization of BAP1, we transduced WT or mutant BAPI constructs into the MM cell with BAPI deletion (Y-MESO-25 cell line) and examined their cellular localization (Fig. 2b). As expected, WT BAPI was preferentially found in the nucleus, whereas mutant BAPI proteins, which were nonsense (Y724X) or truncating (F679L,F6337) forms, showed impaired nuclear localization with increased cytoplasmic localization. Interestingly, a modest increase in the cytoplasmic localization of BAP1 with A95D, a point mutation in the UCH domain, was also observed (Fig. 2c,d).

BAP1 suppresses MM cell proliferation. To determine whether BAP1 has growth-suppressive activity against MM cells, we carried out cell proliferation assays. Transduction of the WT BAPI vector inhibited cell proliferation of the Y-MESO-25 MM cell line with BAPI deletion by approximately 50% compared to the cells with control vector (GFP) (Fig. 3a). In contrast, the mutant BAPI constructs showed reduced or no inhibitory effects according to the possible deterioration effects of the mutants (Fig. 3a). With the colony formation assay, we also found that WT BAPI suppressed the anchor-
Table 1. Summary of BAP1 mutations in 25 malignant mesothelioma cell lines

| Cell line         | Race        | Histological subtypes | Mutation                          | Amino acid change |
|-------------------|-------------|-----------------------|-----------------------------------|-------------------|
| NCI-H28           | Caucasian   | Epithelial            | C.438-24_438-2del23†               | P.r146afs48†      |
| NCI-H290          | Unknown     | ND                    |                                   |                   |
| NCI-H2052         | Caucasian   | Epithelial            | WT†                               |                   |
| NCI-H2373         | Unknown     | ND                    | WT†                               |                   |
| NCI-H2452         | Caucasian   | Epithelial            | C.284c>a†                         | P.a95d†           |
| MSTO-211H         | Caucasian   | Biphasic              | WT†                               |                   |
| ACC-MESO-1         | Japanese    | Epithelial            | WT                                |                   |
| ACC-MESO-4         | Japanese    | Epithelial            | C.2172delc                        | P.y724x           |
| Y-MESO-8D         | Japanese    | Biphasic              | WT                                |                   |
| Y-MESO-9           | Japanese    | Epithelial            | C.2035_2036del2                   | P.f679fsx37       |
| Y-MESO-12          | Japanese    | Epithelial            | WT                                |                   |
| Y-MESO-14          | Japanese    | Biphasic              | C.349_359del11                    | P.k116gsx5        |
| Y-MESO-21          | Japanese    | Epithelial            | WT                                |                   |
| Y-MESO-22          | Japanese    | Biphasic              | WT                                |                   |
| Y-MESO-25          | Japanese    | Epithelial            | Hd (exon13-17) Del 417-729        |                   |
| Y-MESO-268         | Japanese    | Lymphohistiocytoid    | WT                                |                   |
| Y-MESO-27          | Japanese    | Epithelial            | WT                                |                   |
| Y-MESO-28          | Japanese    | Epithelial            | WT                                |                   |
| Y-MESO-29          | Japanese    | Epithelial            | WT                                |                   |
| Y-MESO-30          | Japanese    | Epithelial            | WT                                |                   |
| Y-MESO-45          | Japanese    | ND                    | WT                                |                   |
| Y-MESO-48          | Japanese    | Epithelial            | WT                                |                   |
| Y-MESO-61          | Japanese    | Epithelial            | C.580_581ins40                    | P.p195fsx5        |
| Y-MESO-72          | Japanese    | Epithelial            | WT                                |                   |
| Y-MESO-76          | Japanese    | Epithelial            | WT                                |                   |

†Reported by Bott et al. (2011). −, No change; HD, homozygous deletion; ND, not determined.

The mutation statuses of neurofibromatosis type 2 (NF2), large tumor suppressor homolog 2 (LATS2), Salvador homolog 1 (SAV1), and cyclin-dependent kinase inhibitor 2A (CDKN2A) were previously reported. NLS, nuclear localization signal; UCH, ubiquitin COOH-terminal hydrolase.

age-independent cell growth of the MM cells with BAP1 deletion, whereas mutant BAP1 showed little or no reduced effect (Fig. 3b,c).

The Y-MESO-25 cell line also has the NF2 mutation, which was suspected to influence the growth-suppressive effects of BAP1. We introduced BAP1 vectors into the ACC-MESO-4 cell line, which harbors mutant BAP1 but WT NF2. We confirmed the significant growth-suppressive effects of WT BAP1 (approximately 40%) (Fig. S2), which was quite similar to the results observed in Y-MESO-25 cells (Fig. 3a). Because both WT and mutant BAP1 showed consistently similar growth-suppressive effects regardless of NF2 mutation status, we think a possible growth-suppressive mechanism of BAP1 may be independent of the NF2 pathway.

BAP1-induced gene expression profile. BAP1 has been suggested to have multiple functions, including gene transcription. To determine whether WT BAP1 induces specific gene expression that is involved in the suppression of MM cell proliferation, we carried out a microarray analysis. With the transduction of WT BAP1 into two BAP1-mutated MM cell lines, we found that four pathways were significantly upregulated in both cell lines (Table S1). They were related to the extracellular region, integral to plasma membrane, cytokine activity, and inflammatory response (Tables S2, S3). As eight genes with altered expression, which were involved in these four pathways, were found by using the GenMAPP/MAPP-Finder software, we used quantitative RT-PCR analysis and confirmed the up- or downregulation of these genes (Fig. S3).

These results suggested that some of these pathways might be involved in the growth-suppressive effects of MM cells and BAP1 expression might influence the expressions of these genes. However, well-known strong growth-suppressive pathways or genes that have been defined in other common malignancies were not picked up in these analyses. Thus, these data suggest that the BAP1-regulated pathways may not have a strong growth-suppressive effect individually, which may require cumulative inactivation of the multiple pathways for effective growth-suppression of MM cells.

Irradiation of MM cells induces phosphorylation of BAP1. As one of the major cellular functions of BAP1 is thought to be DNA repair, we induced DNA damage in MM cells with X-ray irradiation to reveal how BAP1 is involved in DNA repair in MM cells. First, when MeT-5A cells, which harbor WT BAP1, were irradiated, we observed the strongest BAP1 phosphorylation level 1 h after IR, which then gradually decreased (Fig. 4a). The phosphorylation level of WT BAP1...
Fig. 2. Effects of BRCA1-associated protein 1 (BAP1) mutation on its own nuclear localization. (a) Endogenous WT BAP1 in NCI-H290 showed nuclear localization, whereas mutant BAP1 (p.F679LfsX37) in Y-MESO-9 showed cytoplasmic translocation. (b) Protein expression of exogenously transduced WT or mutant BAP1 (A95D, Y724X, and F679LfsX37) vectors into the malignant mesothelioma cell line with BAP1 deletion (Y-MESO-25). (c) Immunofluorescence analysis of subcellular BAP1 localization with exogenously transduced BAP1 vectors into the Y-MESO-25 cells. (d) Percentages of subcellular localization (c) were calculated. Wild-type BAP1 was mainly localized in the nucleus (N), whereas BAP1 mutants showed translocation in the cytoplasm (C). HD, homozygous deletion.
was also confirmed to be increased in proportion to IR doses (Fig. 4b). In addition, with immunofluorescence analysis, the strong phosphorylation of the exogenously transduced WT BAP1 into the MM cell with BAP1 deletion was also observed in the nucleus after IR exposure, in the same manner as with endogenous BAP1 (Fig. 4c). Using the MM cell with BAP1 deletion to confirm these results, we then carried out cellular fractionation and found that phospho-mutant-type BAP1 proteins tended to localize in the nucleus after IR like WT BAP1, whereas total mutant-type BAP1 proteins dominantly localized in the cytoplasm (Fig. 4d). These results suggested that several types of mutant BAP1 proteins as well as the WT form might be recruited to IR-induced DSB sites, although the precise mechanism is yet to be determined.

BAP1 stabilizes BRCA1 protein and restores cell survival rates of MM cells with BAP1 deletion after IR. To determine the roles...
of BAP1 on DNA repair in MM cells, we next studied the expression levels of BAP1, and BRCA1, a DNA repair protein that has been suggested to associate with BAP1. We found that the expression levels of BRCA1 protein decreased in more BAP1-mutant cell lines (4 [57%] of 7) than BAP1-WT cell lines (6 [35%] of 17), although it was not statistically significant (Figs 5a and S4). As real-time RT-PCR analysis indicated that BRCA1 mRNA expression levels in the three BAP1 mutant cell lines was the same as with MeT-5A cells and the levels in the other four were at least 50–80% (Fig. 5b), these results suggested a possibility that BAP1 might be involved in the regulation of BRCA1 protein stability. To confirm this hypothesis, we transduced WT BAP1 into the MM cells with BAP1 deletion, which express a very small amount of BRCA1. Noticeably, we found that exogenous BAP1 significantly increased the BRCA1 protein level (Fig. 5c). Furthermore, we found that all three mutant BAP1 forms increased BRCA1 protein levels (Fig. 5c). In addition, regarding subcellular localization of BRCA1, we carried out an immunofluorescence analysis, and found that the subcellular location of BRCA1 proteins was not changed in cells by mutant BAP1 transduction (Fig. S5). In other words, while mutant BAP1 protein preferentially localizes in the cytoplasm, BRCA1 protein keeps residing in the nucleus of BAP1-mutant cells. This suggests that BAP1, regardless of WT or mutant form, may not be a strong determinant for subcellular localization of BRCA1.

Jensen et al. (17) previously suggested that BAP1 enhanced BRCA1-mediated inhibition of cancer cell growth, although they did not directly establish the possibility that BAP1 enhances the BRCA1 protein level. We hypothesized that BAP1 might deubiquitinate BRCA1 and protect it from proteasome-mediated degradation; this might explain the BRCA1 stabilization by the mutant BAP1 forms that retained the DUB activity. We used a proteasome inhibitor, MG-132, to confirm this idea, and found that the BRCA1 protein level was increased after MG-132 treatment (Fig. 5d, arrowhead). This result supported the idea that BRCA1 protein expression might be negatively regulated by the proteasome system, which is suppressed by the DUB enzyme activity of BAP1. In addition, the level of phospho (activated)-BRCA1 decreased in
Y-MESO-25 in comparison with BAP1 WT cell lines (Fig. 5e), and this reduction of phospho-BRCA1 may impair the activity of DNA repair. Finally, we examined whether or not BAP1 affects the survival rates of MM cells that were impaired by IR. We found a significantly low survival rate of BAP1-deleted cells (GFP) compared to the MeT-5A cells (Fig. 5f). However, the WT BAP1 had a significantly strong activity to restore the survival rate of the MM cells with BAP1 deletion up to the same level as MeT-5A cells. Furthermore, we found that the mutant BAP1 of Y724X restored the survival rates significantly compared to GFP control at both 1 and 3 Gy. Meanwhile, BAP1 mutants of A95D and F679Lfs37X showed only marginal restoration (significant only at 1 Gy). These results indicate that WT BAP1 is one of the important components of the DNA repair pathway and even that some mutant BAP1 proteins retain the activity of this pathway in MM cells.

Discussion

In this study, we analyzed BAP1 mutations using a total of 25 MM cell lines, including 19 that we established, and found that 7 (28%) cell lines had an inactivating mutation. We examined the differences between WT and mutant form BAP1 in terms of their cellular functions, including subcellular localization, growth-suppressive activity, and cellular resistance against IR. We showed that, even though several mutant forms lack growth-suppressive activity, they preserve some functional activities of BAP1 in vitro.

It is worth noting that BAP1 can restore the protein expression of BRCA1. Although BRCA1 expression significantly decreased in an MM cell line with BAP1 deletion, Y-MESO-25, the transduction of the WT and even mutant forms of BAP1 restored the survival rates of MM cells with BAP1 deletion impaired with IR. *P < 0.01 and **P < 0.05 versus empty lentivirus control (GFP).
BAP1 inhibits the E3 ligase activity of BRCA1 in a manner independent of its deubiquitination activity. Hence, it will be necessary to determine the involvement of deubiquitination activity of BAP1 for BRCA1 stabilization in detail in a future study.

The MM cells with BAP1 deletion transduced with WT BAP1 showed a good survival rate that was comparable to Met-5A at 1-Gy IR. This result strongly suggests that the recovery of the MM cells with BAP1 deletion from the IR damage is attributable not only to the exogenous expression of BAP1 itself but also to the upregulation of BRCA1. In addition, the MM cells with BAP1 deletion transduced with mutant BAP1 (Y724X) tended to have a better survival rate than the cells with control vector (GFP). This result supports the idea that some BAP1 mutants still partially retain important functions of BAP1 that affect cellular viability; in this experiment, mutant BAP1 contributed to the recovery of the DNA repair system together with the upregulation of BRCA1.

We speculated that several distinct domains are particularly important for the tumor suppressor activity of BAP1. For example, Ventii et al. (30) showed that the NLS was necessary for BAP1 to act as a tumor suppressor. They used an in vivo implantation experiment with a BAP1-null human lung cancer cell line that was transduced with BAP1 vectors, and found that WT BAP1, but not mutant BAP1 lacking the NLS, showed significantly weaker tumor development. We also observed that NLS-lacking F679Lfs mutant BAP1, which was dominantly localized in the cytoplasm, had a significantly inferior growth-suppressive effect compared to the WT BAP1.

Mashtalir et al. (33) also underscored the functional importance of the UCH-domain. While ubiquitination enzyme UBE2O monoubiquitinates the NLS of BAP1 and induces translocation (inactivation) of BAP1 from the nucleus to the cytoplasm, DUB activity of the UCH-domain of BAP1 counteracts the UBE2O activity mediated through the intramolecular interaction between the UCH-domain and COOH-terminal domain of BAP1. Thus, the autodeubiquitinating activity of the UCH-domain is crucial to its growth-suppressive function. Consistent with this, we found that the A95D mutation within the UCH-domain of BAP1 showed reduced growth-suppressive activity.

Additionally, it was intriguing to find that phosphorylation of mutant BAP1 seemed to induce its translocation from the cytoplasm to the nucleus, as Western blot analysis detected strong p-S592 BAP1 in the nucleus of the mutant F679LfsX37-transduced cells (Fig. 4d, second lane from the right). We initially thought that this observation might have resulted from active transportation of phosphorylated BAP1 protein. As this BAP1 mutant form lacks NLS, we thought that this active transportation might have been induced in an NLS-independent manner. However, we currently think that this observation does not necessarily reflect nuclear transport of the phosphorylated mutant BAP1. As a fraction of the F679LfsX37 X BAP1 mutant protein resides in the nucleus (Fig. 4d, fourth lane from the right), even if much less abundant than in the cytoplasm, the remarkably increased p-S592 BAP1 signal of F679LfsX37 may just reflect the phosphorylation of the mutant BAP1 that has been localized in the nucleus from the beginning. In any event, the phosphorylation effect of BAP1 must be clarified in more detail in terms of changes in the activity and cellular localization of both WT and mutant BAP1.

In conclusion, the present study suggests that various types of BAP1 mutations as well as WT BAP1 may exert different levels of growth suppression and/or DNA repair activities with different levels of residual functions. This result indicates that various BAP1 mutations in MM cells may cause a different sensitivity to chemotherapy and/or radiation therapy. Therefore, our study suggests that BAP1 mutation status must be precisely determined in order to select an optimal therapeutic strategy for individual patients with MM.

Disclosure Statement

The authors have no conflict of interest.

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