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Subcellular Localization of BRCA1-Associated Protein (BAP1) in Uveal Melanoma Cases and Assessment of Its Functions

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Keywords

- BRCA1-associated protein
- BAP1
- subcellular localization

Abstract

Background: BRCA1-associated protein 1 (BAP1) is a nuclear-localized deubiquitylase (DUB) that belongs to the ubiquitin carboxy terminal hydrolase family of DUBs. BAP1 acts as a tumor suppressor inactivated in various cancers. Several studies in cancer cells containing BAP1 mutations indicated that BAP1 nuclear localization and catalytic activity are required for its growth and suppressive properties. **Methods:** Subcellular localization of BAP1 was studied in B-16 cells (WT BAP1), as well as 15 fresh frozen samples of Uveal Melanoma (UM) cases using immunofluorescence staining and confocal microscopy imaging. Wound Healing Assay was used to evaluate the role of BAP1 in the regulation of cell migration in B-16 cells. Clonogenic Assay was used to assess cell proliferation and the effect of BAP1 on DNA repair after irradiation (IR).**Results:** WT BAP1 was mostly nuclear and small amount was cytoplasmic. Among 15 UM samples 2 showed nuclear localization, while 4 showed only extranuclear BAP1. In 3 samples BAP1 was both nuclear and extranuclear, while 6 specimens appeared with no BAP1. In wound healing assay, BAP1 stimulated cell migration, while BAP1 knock-out slowed it down. In clonogenic Assay, more proliferation rate was detected in B-16 cells colonies with WT BAP1 even after IR confirming the role of BAP1 in cell proliferation and DNA repair. **Conclusion:** Mutant BAP1 is localized in a different way in the same type of cells (nuclear or cytoplasmic or absent). BAP1 is important for cell proliferation, migration, and DNA repair.

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Introduction

BRCA1-Associated Protein 1 (BAP1), encoded by the BAP1 gene on chromosome 3p21.1, was originally discovered by Jensen and colleagues in 1998 as an ubiquitin carboxy terminal hydrolase which is responsible for removing ubiquitin from proteins. [1,2]

Ubiquitination is a post-translational modification that influences nearly all eukaryotic cellular processes. In this process, ubiquitin is covalently conjugated to targeted proteins, and so regulates or modifies protein's biological activity, stability or its subcellular localization.[3]

Deubiquitination, performed by deubiquitinating enzymes (DUBs), is the approach that regulates ubiquitin modified proteins through removing ubiquitin modification. BAP1 is a nuclear-localized DUB that was originally identified as a protein interacting with the breast cancer susceptibility gene product (BRCA1). [4]

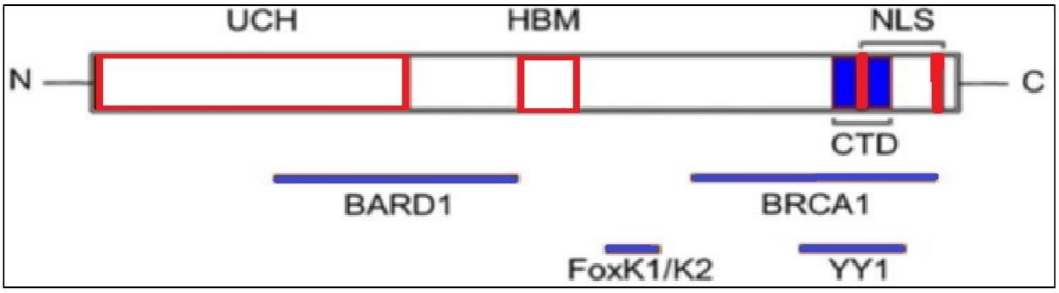

Fig. (1): Functional domains of BAP1. BAP1 consists of 729 amino acids. ubiquitin carboxyl hydrolase domain (UCH)(1–250 aa.); HBM binding domain (365–385aa.); CTD (C-terminal domain) (635–693 aa.); nuclear localization signals (NLS) (656–661 and 717–722 aa.); BARD1 binding region (182–365 aa.); FoxK1/K2 binding region (477–526 aa.); BRCA1 binding region (596–721 aa.) and Ying Yang 1 binding region (YY1) (642–686 aa.) [5]

Wild type BAP1 consists of 729 amino acids. It includes a ubiquitin carboxy-terminal hydrolase (**UCH**) domain located at its N-terminal region (1–240) [1,6]. BAP1 contains 2 nuclear localization signals (**NLS1, NLS2**) at its C-terminal region, (656–661 and 717–722). [4] BAP1 also contains binding regions through which it interacts with host cell factor 1 (HCF-1), BARD1, FoxK1/K2, BRCA1, Ying Yang 1 (YY1) and ASXL1/2. [5,7]

BAP1 is responsible for many processes that have a role in carcinogenesis. It is a multifunctional tumor suppressor protein that has various functions. BAP1 involvement in certain multiprotein complexes is important to regulate the control of cell cycle and proliferation.[2] The complex of YY1, HCF-1, E2F1, and BAP1 as a transcriptional factor is recruited to different promoters to induce upregulation of the genes responsible for cell proliferation.[8] Other suggested functions for BAP1 include chromatin remodeling and regulation of: gene expression, DNA damage, apoptosis, cellular metabolism and, immune response.[9] It is unclear whether BAP1 mutations in cancer cells affect its function or not. [4]

At first, BAP1 was demonstrated to localize into the nucleus where it binds primarily to BRCA1 and functioning as a tumor suppressor. [2] In subsequent years, several research groups have showed that BAP1 protein is localized in the nucleus and the cytoplasm. It was also found that truncating mutations impair BAP1 nuclear translocation, due to the presence of nuclear localization signals at the C-terminal region of BAP1.[10,11]

UBE2O (ubiquitin-conjugating enzyme) causes BAP1 accumulation in the cytoplasm by multi-monoubiquitination of its NLS signals. BAP1 regulates its nuclear localization and counteracts the previous mechanism through intra-molecular auto-deubiquitination of the NLS sites[11]. It is thought that BAP1 may exert some cytoplasmic activities due to the presence of a well-established mechanism that keeps BAP1 in the cytoplasm. [12]

Bononi et al. (2017) discovered that BAP1 binds, deubiquitinates, and stabilizes type 3 inositol-1,4,5-trisphosphate receptor (IP3R3) in the endoplasmic reticulum (ER). This, in turn, modulates calcium (Ca2+) release from the ER into the cytoplasm and mitochondria and promotes apoptosis. This suggests that BAP1 has independent tumor suppressor functions in the cytoplasm and nucleus. [9]

There is evidence that BAP1 mutations are found in a common array of tumors, many of them show aggressive tumor phenotypes and have a poor response to standard treatments.[2] Clinical studies have revealed that BAP1 is frequently lost or inactivated in many cancers.[13] Many studies have indicated that germline mutations of BAP1 causes a tumor predisposition syndrome (BAP1-TPDS), a hereditary cancer syndrome that is characterized by a high incidence of early-onset malignancies including malignant mesothelioma, cutaneous melanoma, uveal melanoma, and several other cancers. [2]

In most tumor cells with mutated BAP1, there is no nuclear staining nor cytoplasmic staining. At times, a mutated inactive BAP1 accumulates in the cytoplasm where it forms amyloid, and it may show cytoplasmic staining without associated nuclear staining. As mutations in Ubiquitin Proteosome System (UPS) genes, including BAP1, have been implicated in many cancers, so the UPS has emerged to be used in many anticancer drugs. [1,14]

This study aimed at the assessment of subcellular localization of wild type BAP1 protein in B-16 cell line, as well as in specimens of uveal melanoma tumors. The impact of the presence and absence of BAP1 protein on its different functions, such as DNA repair, cell proliferation and migration was also investigated.

Materials and methods:

This study was approved by Institutional Review Board (IRB), Faculty of Medicine, Mansoura University (code number: MDP.18.07.08). All lab work took place in College of Medicine, Ohio State University, USA.

1. **Cell lines**

B16 F10 (melanoma cells), devoid of BAP1, were obtained from ATCC (American Type Culture Collection). Cells were transfected with WT BAP1 and used for both subcellular localization and functional study.

BAP1 knock-out cell lines were generated using CRISPR-Cas9 and were also used for functional study.

2. **Subcellular localization of BAP1**

B-16 cells transfected with WT BAP1, as well as 15 fresh frozen samples of Uveal Melanoma with various BAP1 mutations were studied using immunofluorescence staining and confocal microscopy imaging.

Immunofluorescence Staining:

Materials

Primary antibodies

1. **Mouse anti BAP1 (C-4)**: Catalog #sc-28383 from Santa Cruz Biotechnology was used as a marker for BAP1
2. **GM130 (D6B1) XP® Rabbit mAb** (Catalog #12480) from Cell Signaling Technology was used as a marker for Golgi.

Secondary antibodies

1. **Alexa Fluor 488-conjugated goat antimouse polyclonal IgG** (dilution, 1:1000), (Catalog # A-11001) Thermo Fisher Scientific, Inc (Pittsburgh, PA)
2. **Alexa Fluor 633-conjugated goat antirabbit polyclonal IgG** (dilution, 1:1000), (Catalog # A-21071) Thermo Fisher Scientific, Inc (Pittsburgh, PA)].

ProLong Gold antifade reagent with DAPI (4',6-diamidino-2-phenylindole) (Catalog #P36931) from Thermo Fisher Scientific, Inc (Pittsburgh, PA) was used for nuclear counterstaining.

Technique

The slides were fixed using 3% paraformaldehyde for 10 min, then were incubated with 0.1% Triton X-100 in PBS for 10 minutes to permeabilize the membranes. Then, slides were blocked using Blocking Buffer (BB) with Goat IgG for 1 hour, followed by incubation with primary antibody for 1 h at RT. The slides were coated with 100% Normal Goat serum for 20-30min, then incubated in 2ry antibody for 1 h in dark. Lastly, the slides were mounted with ProLong Gold antifade reagent with DAPI. After each step, the slides were washed (3times, each one is 5 min) in PBS.

Imaging:

All images were taken using Olympus Spectral Inverted FV3000 Confocal microscopy (catalog # FV3000) Evident Olympus (Waltham, MA, United States) equipped with a 60X oil objective at OSU CMIF [Ohio State University Campus Microscopy & Imaging Facility] using the appropriate laser. Images were analyzed using FV3000 viewer Software.

3. Functional study

a) Wound Healing (or Scratch) Assay

This technique was used to evaluate the role of BAP1 in the regulation of cell migration and invasion.

Method

One day before, cells were seeded in 6 well plates to which 2 mL of Opti-MEM growth medium was added. Once at confluence (18-24 hours), the cell layer was scratched using a pipette tip. the tip was kept perpendicular to the base of the plate. Then, the cells were gently washed to remove detached cells, then replenished with fresh medium. Phase contrast microscope was used for imaging on 100x magnification. The cells were placed in incubator and imaged every 12 hours until they migrate to meet in the middle. An empty vector was used as control.

b) **Clonogenic Assay**

This technique was used to assess cell proliferation. It was also used to assess the effect of BAP1 on DNA repair after treatment with ionizing radiation (IR).

Method

One day before, cells were plated in 6-well plates to which 2 mL of Opti-MEM growth medium was added. Then, some plates were irradiated with 7.5Gy ionizing radiation dose, then incubated for 7

days. The surviving colonies were washed with PBS and fixed with 10% buffered formalin (4% formaldehyde) for 20 min. Then cells were stained with crystal violet for 10 min, followed by PBS washes. Colonies were counted in 3 randomly selected fields and assessed by Image J software (National Institutes of Health, Bethesda, MD). Counting was done after 1, 3, 6 days.

Results:

A. Results of subcellular localization of BAP1:

WT BAP1 in B-16 cells was mostly nuclear and small amount was cytoplasmic and did not colocalize with Golgi. **(Fig.2)**

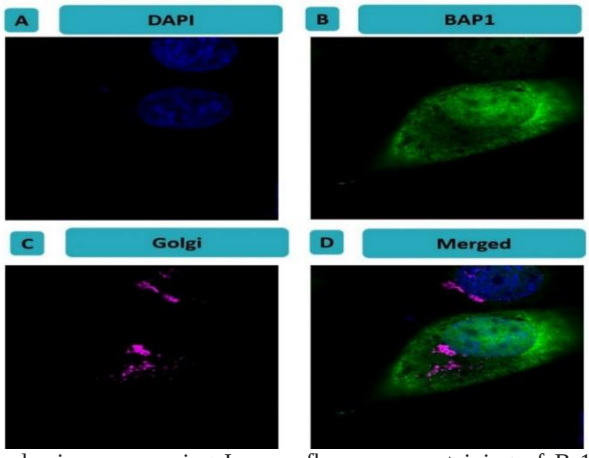

Fig. (2): Photomicrograph of confocal microscopy using Immunofluorescence staining of B-16 cells transfected with WT BAP1 showing A**)** nuclei stained blue with DAPI, B) BAP1 stained green, C) Golgi apparatus stained magenta, D) Merged image showing the overlapping to show localization of the WT BAP1, it appears mostly nuclear and small amount is extranuclear. Magnification ×600.

Among 15 UM samples, two (2/15) (13.3%) showed nuclear localization. Four specimens (4/15) 26.6% showed only extranuclear BAP1 that colocalized with Golgi and in three specimens (3/15) 20% BAP1 was both nuclear and extranuclear but was not colocalized with Golgi. Six of the fifteen specimens (6/15) (40%) appeared with no BAP1, neither nuclear nor cytoplasmic. **(Fig.3)**

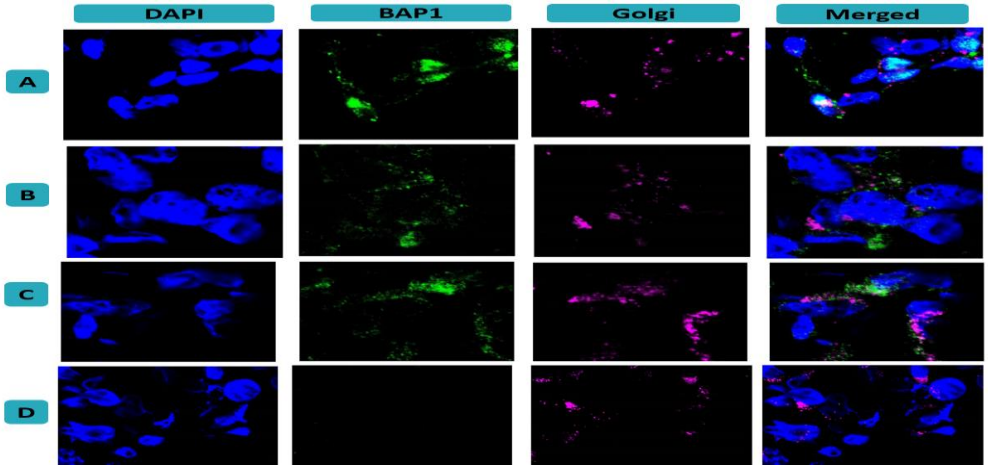

Fig. (3): Photomicrographs of confocal microscopy using Immunofluorescence staining of Uveal Melanoma specimens. Nuclei stained blue with DAPI, BAP1 stained green, Golgi apparatus stained magenta, merged images show the overlap to demonstrate the localization of the BAP1. A**)** BAP1 is mostly nuclear and small amount extranuclear. B) BAP1 is nuclear and cytoplasmic but not colocalized with the Golgi. C) BAP1 is mostly extranuclear and colocalized with the Golgi. D) NO BAP1 appears all over the cells. Magnification ×600.

B. Results of Wound Healing (or Scratch) Assay

The images revealed that BAP1 stimulated B-16 cell migration while BAP1 knock-out slowed down the migration and invasion of B-16 cells. After 12 hours, the distance between wound edges was less in cells with WT BAP1 than BAP1 knock-out cells and empty vector cells. After 24 hours, the space was full of cells in WT BAP1 cells, while there was a distance between wound edges of BAP1 knock-out cells and wider distance in empty vector cells. **Fig (4)**

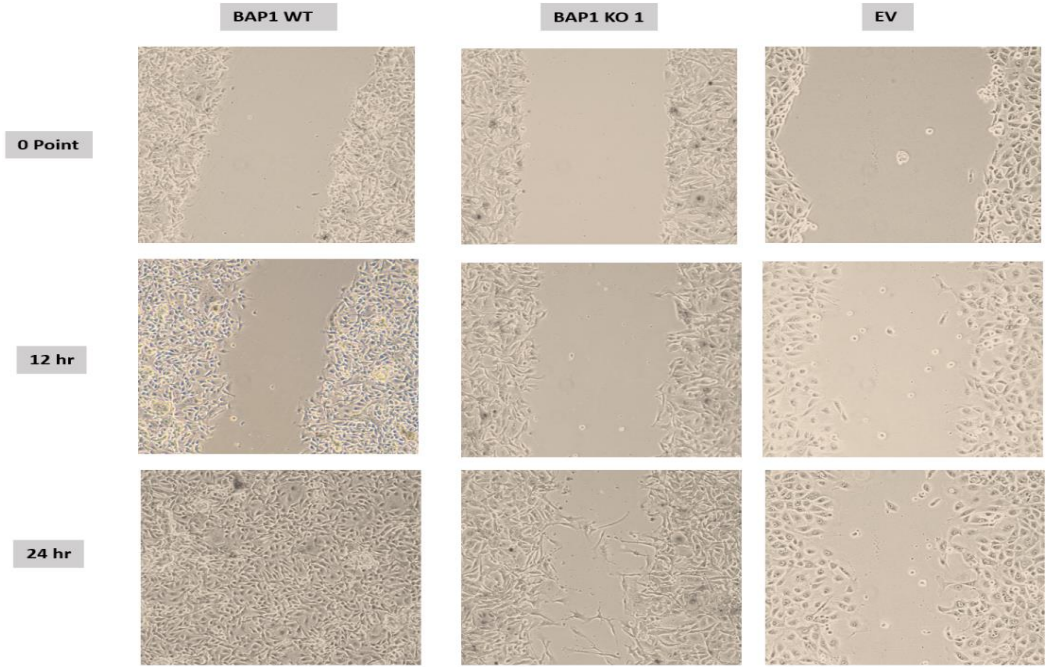

Fig (4) Wound healing assay. Photomicrograph of phase-contrast microscope showing the wound closure rate of cells among different groups of B-16 cells transfected with WT BAP1 and cells with BAP1 knockout clone (KO1). The images were taken at 0 point, 12 hours and 24 hours. After 12 hours, the distance between wound edges was less in cells with WT BAP1 than BAP1 KO1. After 24 hours, the space is full of cells in WT BAP1 cells, while there is a distance between wound edges of BAP1 KO1. WT; Wild type BAP1, KO1; BAP1 knockout clone1, EV; empty vector. Magnification ×100.

C. Results of Clonogenic Assay

WT BAP1 colonies appeared denser in color indicating more proliferation rate of WT BAP1 colonies than BAP1 knockout colonies **Fig (5)**.

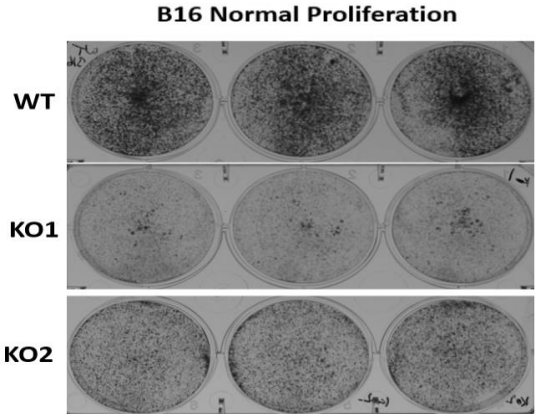

Fig (5) Clonogenic Assay; a representative micrograph of (Day 3) colonies of B-16 cells transfected with WT BAP1and BAP1 knockout (KO) clones (KO1 & KO2), the plates are stained with crystal violet stain. colonies with WT BAP1 are denser in color indicating more proliferation than colonies with BAP1 knockout (KO) clones

Both linear and Column bar graphs were used to demonstrate the relation between percentage of B-16 colonies proliferation at 0,1,3 and 6 days. The graphs confirmed that the number of WT BAP1 colonies was more than colonies of BAP1 KO clones at day1 & day 3. While at day 6, there was an increase in KO2 clone to be slightly more than WT colonies **Fig (6)**.

According to Fig (6):

At 0 point: No significant difference was detected between WT clone (19.54 ± 2.63), KO1 clone (19.82 ± 2.14) and KO2 clone (19.9 ± 2.40).

At day 1: Non-significant (P = 0.216) decrease was detected between KO1 clone (**20.74** ± 2.86) and KO2 clone (٢٢,٢٩ ± 3.19) versus WT clone (24.03 ± 3.09).

At day 3: A highly significant (P < 0.001) decrease of B16 cells was found between KO1 clone (25.36 ± 3.77), and KO2 clone (36.34 ± 4.57) versus WT clone (50.78 ± 6.81), and also between KO1 clone and KO2 clone.

At day 6: Non-significant (P = 0.084) decrease of B16 cells was found between KO1 clone (58.34 ± 7.73) and WT clone (63.71 ± 8.16), while non-significant increase was observed between KO2 clone (66.71 ± 7.52) and WT clone.

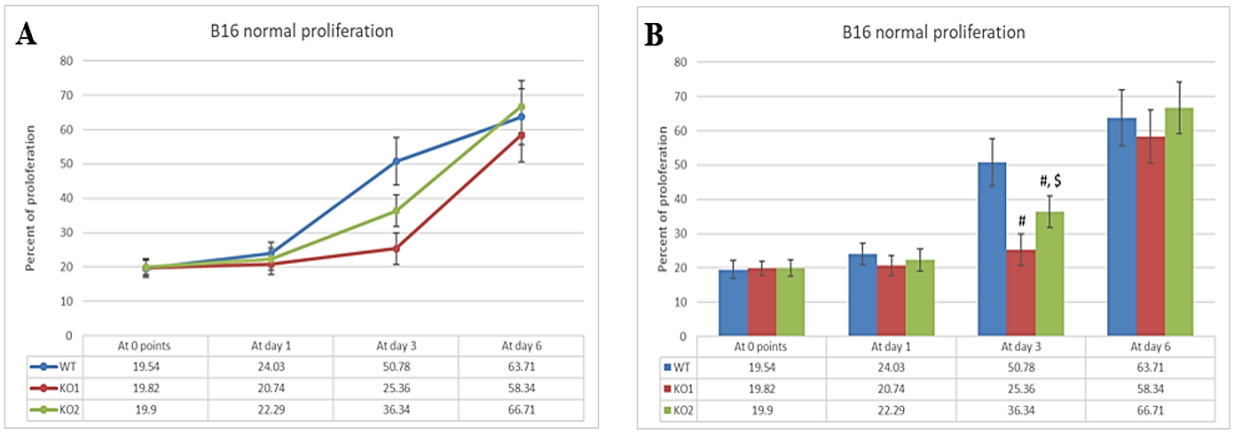

Fig (6) A) Linear graph and B) Column bar graph showing the relation between percentage of B-16 colonies proliferation at different days of the clonogenic assay. WT; Wild type BAP1, KO1; BAP1 knockout clone1, KO2; BAP1 knockout clone 2. Both graphs show the difference in cell proliferation between WT BAP1 and BAP1 knockout clones. Cells with WT BAP1 are significantly more in number than KO clones at day1 & day 3. While at day 6 there is an insignificant increase in KO2 clone to be more than WT colonies. **#:** Significance in relation to WT group. **$:** Significance in relation to KO1 group.

After irradiation exposure with 7.5Gy ionizing radiation (IR) dose, B-16 colonies with WT BAP1 appeared denser in color than colonies with BAP1 knockout (KO) clones, indicating more proliferation rate of colonies with WT BAP1 and confirming its role in DNA repair **Fig (7)**.

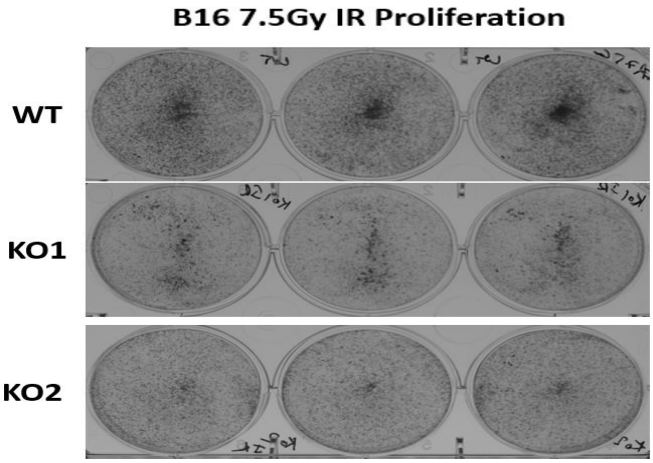

Fig (7) Clonogenic Assay; a representative micrograph of colonies of (Day 3) B-16 cells transfected with WT BAP1and cells with BAP1 knockout (KO) clones (KO1 & KO2) after irradiation radiation (IR) exposure with 7.5Gy dose. The plates are stained with crystal violet stain. Colonies with WT BAP1 after IR are denser in color indicating more proliferation rate of colonies with WT BAP1 than colonies with BAP1 KO clones which proves the importance of BAP1 in DNA repair

Linear and Column bar graphs were used to demonstrate the relation between percentage of B-16 colonies proliferation after IR at 0,1,3 and 6 days of the clonogenic assay. The graphs showed that the number of WT BAP1 colonies was more than BAP1 KO clones at all durations **Fig (8)**.

According to Fig (8):

At 0 point: No significant difference was found between WT clone (20.32 ± 2.63), KO1 clone (19.43 ± 2.14) and KO2 clone (19.98 ± 2.40).

At day 1: Non-significant (P = 0.246) decrease of B16 cells was detected between KO1 clone (20.97 ± 2.86) and KO2 clone (21.57 ± 3.19) versus WT clone (23.47 ± 3.09).

At day 3: A highly significant (P < 0.001) decrease of B16 cells was recorded between KO1 clone (23.55 ± 3.77), and KO2 clone (27.31 ± 4.57) versus WT clone (37.87 ± 6.81).

At day 6: A highly significant (P < 0.001) decrease of B16 cells was found between KO1 clone (34.50 ± 7.73), and KO2 clone (36.49 ± 7.52) versus WT clone (50.34 ± 8.16).

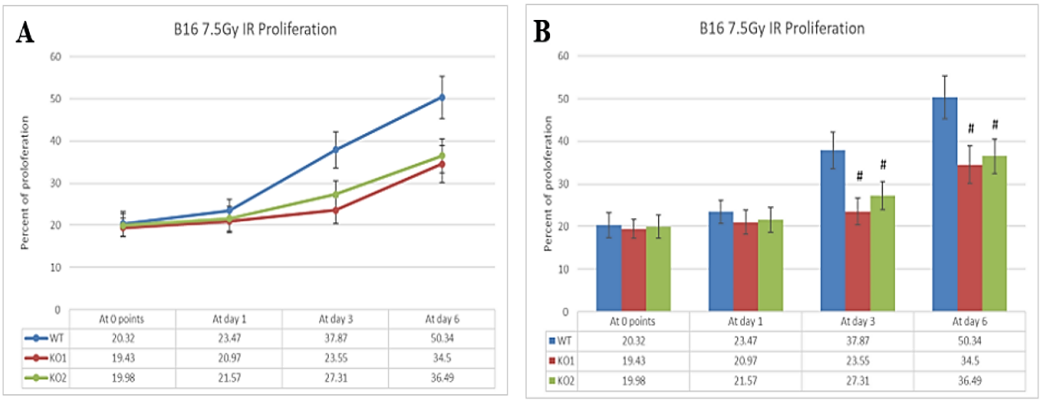

Fig (8) A) Linear graph and **B)** Column bar graph showing the relation between percentage of B-16 colonies proliferation at different days of the clonogenic assay after irradiation exposure with 7.5Gy ionizing radiation (IR) dose. WT; Wild type BAP1, KO1; BAP1 knockout clone1, KO2; BAP1 knockout clone 2. Both graphs show the difference in cell proliferation between cells with WT BAP1 and BAP1 knockout clones after IR. The cells with WT BAP1 are significantly more in number than the KO cells at day 3& 6 which proves the importance of BAP1 in DNA repair. **#:** Significance in relation to WT group.

Discussion:

BAP1 subcellular localization was thought be important for its tumor suppressive activity. Initially, the BAP1 tumor suppressive effects were correlated to its nuclear localization, suggesting that DUB activity of BAP1 predominantly regulates nuclear targets that are involved in gene transcription and related cellular processes. Recently, BAP1 was demonstrated to participate in cellular calcium release and act as tumor suppressor in the cytoplasm. This observation suggests that BAP1 might exert distinct functions in the different subcellular compartments.[7,9]

The present study revealed that WT BAP1 was mostly nuclear and only a small amount was cytoplasmic which was not localized at Golgi apparatus. Similarly, **Hakiri et al. (2015)** [15] and **Bhattacharya et al. (2015)**[16] found that WT BAP1 (in malignant mesothelioma and human lung cancer cell lines respectively) showed endogenous BAP1 expression mainly in the nucleus and small amount was cytoplasmic. On the contrary, **Baas et al. (2021)** found that WT BAP1 was mainly nuclear with no cytoplasmic staining.[13]

There is a clinical importance of BAP1 subcellular localization, and BAP1 immunohistochemistry (IHC) is now an integral diagnostic routine in clinics. The nuclear BAP1 (nBAP1) protein expression prevalence in UM was investigated in several studies to find a correlation with the clinical outcome. Metastatic risk and other features linked to poor prognosis were found to be related to loss of protein expression of nBAP1. [17,18] Significantly, somatic BAP1 loss was observed in 84% of metastatic UM, indicating the important role of BAP1 in cancer progression [19].

In the present work, tumor samples were obtained from 15 uveal melanoma (UM) patients. Fresh frozen tissue specimens were prepared for immunofluorescence studies to detect the BAP1 protein localization. The anti BAP1 (C-4) 1ry antibody was used for BAP1 localization and the GM130 antibody was used for localization of Golgi apparatus.

Two specimens (2/15) (13.3%) demonstrated nuclear localization of BAP1, while four of the fifteen specimens of UM cases (4/15) (26.6%) showed extranuclear BAP1 that colocalized with Golgi. In other three specimens (3/15) (20%) BAP1 was both nuclear and extranuclear but did not colocalize with Golgi. Six of the fifteen (6/15) (40%) specimens appeared with no BAP1, neither nuclear nor cytoplasmic. Similarly, loss of nuclear BAP1(nBAP1) protein expression was observed in many uveal melanoma (UM) cases. The loss of nBAP1 expression is thought to be the strongest prognostic marker in UM, confirming its importance in UM progression. Moreover, researchers noted the presence of differing cytoplasmic staining patterns of BAP1 protein (cBAP1) using IHC suggesting a difference in its cytoplasmic localization.[18,20]

The results of the present work confirm previous data reported by many authors. **Gammon et al. (2013)** observed cytoplasmic BAP1 in 53% of cases of epithelioid Spitz tumors where nuclear BAP1 was negative. Using immunohistochemistry, the authors revealed a high frequency of BAP1 loss in the Spitz tumor component of the neoplasm. 15 of 19 cases showed loss of BAP1 nuclear localization, while 4 cases only showed strong nuclear staining for BAP1. They identified a clumped perinuclear pattern of cytoplasmic

BAP1 in 8 of the 15 cases that lack nuclear expression, , while in 7/19 there was total loss of BAP1 nuclear staining. [21]

Pilarski et al. (2014) revealed that the BAP1 lost its nuclear localization in the tumor cells having germline truncating mutation. On the other hand, stromal cells showed strong expression of BAP1, indicating BAP1 inactivation in the tumor cells. The authors also reported cytoplasmic sequestration of mutated BAP1 protein in patients with germline mutations in BAP1. [22]

Using immunohistochemical staining (IHC), **Koopmans et al (2014)** and **Kalirai et al (2014)** observed loss of nuclear BAP1 protein in 43% and 51% of all uveal melanoma cases, respectively. They also reported the presence of cBAP1 of different staining patterns. [17,18]

Van de Nes et al. (2016) examined 66 UM cases and found that 89% of cases had a BAP1 mutation, and lack of nuclear BAP1 localization at the same time. They suggested that truncated BAP1 protein with loss of nuclear signals and/or reduced protein or mRNA stability were due to BAP1 nonsense or frameshift mutations. [20]

Farquhar et al. (2018) demonstrated 127 specimens of UM cases. 68 (53.5%) were nuclear BAP1 (nBAP1) positive, while in 59 (46.5%) nBAP1 was negative. Nuclear BAP1 positive demonstrated diffuse cytoplasmic staining in 53/68 (77.9%) of cases, while the remaining 15/68 (22.1%) cases did not show focal perinuclear pattern nor cytoplasmic BAP1 expression. Similar to the present results, the authors observed that BAP1 was localized near, or probably within the Golgi in UM cells. They explained that it is possible that the UM cells bypass the premature stop codon by using alternative splice sites and/or read-through translation to generate misfolded proteins that are not able to exit the Golgi following processing. [23]

Carbone et al. (2020) found that large BAP1 deletions in most mesothelioma tumor cells lead to truncating mutations that results in loss of nuclear and cytoplasmic BAP1 staining. [10]

The present results showed that BAP1 gene mutations in UM samples may lead to nuclear loss or total loss of BAP1 protein that may indicate bad prognosis. Thus, BAP1 immunostaining may play a major role in prognostic testing of UM patients, as suggested in previous reports. [20] For example, the absence of nuclear staining of BAP1 is a valuable diagnostic test to differentiate positive nuclear staining of benign mesothelioma from negative nuclear staining of malignant mesothelial cells, including distinguishing benign pleural effusions, benign chronic pleuritis, and benign atypical mesothelial hyperplasia from mesothelioma[10].

To investigate the importance of BAP1 protein in the cells, stable BAP1 knock-out cell lines were generated in the present study using CRISPR-Cas9. This was followed by functional study that involved the scratch-wound assay, clonogenic assay (normal cell proliferation) and clonogenic assay after ionizing radiation (IR) exposure in both WT BAP1 and BAP1 knockout cell lines.

The wound healing assay is a simple assay usually used to measure basic cell migration parameters (speed, persistence, and polarity). Cells are grown to confluence, then the cell monolayer was scratched with a pipette tip. Cells at the edges

proliferate and migrate into the space.[24] Images are taken at 0 point, 12 hours, and 24 hours and the wound closure capacity is determined by photographing.

The results of the present work showed that BAP1 stimulated cell migration, while BAP1 knock-out slowed down the migration and invasion of B-16 cells. After 12 hours, the distance between wound edges was less in cells with WT BAP1 than BAP1 knock-out cells. After 24 hours, the space was full of cells in WT BAP1 cells, while there was a distance between wound edges of BAP1 knock-out cells.

Consistent with the present results, **Matatall et al. (2013)** and **Chen et al.(2019)** found that the knockdown of BAP1in UM cells showed decrease in the cell migration and decrease of its motility in wound healing assays.[25,26] Similarly, **Pan et al. (2015)** and **Jia et al. (2021)** found that BAP1 knockdown inhibited cell migration in the Transwell assay and they suggested that BAP1 may cooperate with other genes and transcription factors to mediate cell invasion and metastasis.[27,28] Also **Chen et al.(2019)** Transwell assay demonstrated that BAP1 KO induced invasion defect.[26]

On the contrary, **Shen et al. (2016)** found that BAP1 might act as suppressor protein of invasion. They performed wound-healing and Transwell cell invasion assays to find an apparent decrease in cell migration by BAP1 overexpression. Meanwhile, silencing of BAP1 gene expression with siBAP1 resulted in early wound healing compared with the controls. The authors used 2 lines of lung cancer cells, and they observed a significant decrease of matrix metalloproteinase-2 (MMP-2) and Vimentin. They also observed remarkable increase of E-Cadherin. They hypothesized that the expression of metastasis related proteins is affected by BAP1 expression in these lines, which influence the metastatic capability of lung adenocarcinoma cells.[29]

Clonogenic assay is used to assess cell proliferation and to investigate the effect of BAP1 on DNA repair following treatment with ionizing radiation (IR).[30]

In this study, colonies of B-16 cells transfected with WT BAP1 and BAP1 knockout (KO) clones were stained with crystal violet. WT BAP1 colonies appeared denser in color, indicating more proliferation rate of WT BAP1 than BAP1 knockout colonies. After colony count and statistical analysis, linear and Column bar graphs confirmed that the number of WT BAP1 colonies was insignificantly more than colonies of BAP1 KO clones at day1 and was significantly more at day 3.

The current results support previous cell proliferation studies. **Matatall et al. (2013)** and **Pan et al. (2015)** found that BAP1 loss decreased cell proliferation using colony formation in soft agar plates stained with crystal violet. [25,27] **Hakiri et al. (2015)** used cell proliferation and colony formation assays and found that MM cell lines with BAP1 deletion suppressed cell growth and cell proliferation by approximately 50%, compared to cells transduced with empty vector.[15]

Liu et al (2018) found that knockdown of BAP1 significantly inhibited cell proliferation. The authors explained that BAP1 inhibition leads to increased level of Histone H2A (H2Aub), which interferes with chromatin and histone modifications at double-strand breaks. Thus,

increased H2Aub is inversely proportional to cell proliferation. [31]

Luo et al. (2021) used B16 cells and found that BAP1 deletion reduces colony-forming capacity of B16 cells and cutaneous melanoma cell lines. They also confirmed their results by re-expression of BAP1 in BAP1 KO cells which rescues growth inhibition in vitro and in vivo.[32]

Masclef et al. (2021) revealed that absence of BAP1 in cells decreases cell proliferation and delays G1 to S transition of cell cycle, indicating that BAP1 has a role in cell-cycle progression by directly acting at the replication fork. They also explained that BAP1 forms a complex with both HCF-1 and YY1 that promotes cell proliferation. [33]

The present clonogenic assay, surprisingly, showed that BAP1 KO1 and KO2 cells increased markedly, although insignificantly, in number at Day 6 and even slightly exceeded WT BAP1 cells (KO2). This can be explained by **He et al. (2019)** who found that the transcription factor *Mitf had* an approximately fourfold increase in expression in BaP1 deficient melanocytes, compared with control melanocytes. The *Mitf* gene is a main regulator gene of melanocyte differentiation and development. It encodes the melanocyte inducing transcription factor (MITF) and its increase results in cell survival that may explain the increased cell proliferation of BAP1 KO2 cells at day 6. [34]

One of the important functions of BAP1 is DNA repair. In the present study, DNA damage was induced in cells with 7.5 Gy ionizing radiation (IR). Then the plates were stained with crystal violet to show how BAP1 participates in DNA repair in B-16 cells transfected with WT BAP1 and BAP1 KO clones.

After IR, B-16 colonies with WT BAP1 in the present work appeared denser in color than colonies with BAP1 knockout (KO) clones. This indicates more proliferation rate of colonies with WT BAP1 and confirms its role in DNA repair. Linear and Column bar graphs showed that the number of WT BAP1 colonies was significantly more than BAP1 KO clones at days 3& 6. In agreement with the present results, **Peña-Llopis et al. (2012)** and **Liu et al (2018)** found that BAP1 deficient cells were more sensitive to ionizing radiation as fewer colonies were formed in clonogenic assays.[31,35]

Bononi et al. (2017) revealed that BAP1 decreased levels lead to enhanced DNA damage (due to decreased activity of nuclear BAP1). However, after IR, their clonogenic assays showed increased cell survival with reduced levels of BAP1 compared with WT BAP1. The authors explained this discrepancy as these cells (with low BAP1 level) lead to decreased apoptosis (due to decreased activity of cytoplasmic BAP1) and therefore, cellular transformation increased (due to decreased activity of both nuclear and cytoplasmic BAP1), and so, increasing the risk of growing into a malignancy. [9]

Bononi et al. (2017) stated that there is a balance between DNA damage and cell death that controls the outcome: the more DNA damage in cells, the higher the risk that cells may grow into malignancy. In some tissues in which Ca2+-induced apoptosis plays a critical role in cellular transformation, the malignancies that arise, are more frequently associated with the BAP1 cancer syndrome. [9]

Recently, **Baas et al. (2021)** explained that the different cell types behave differently in absence of BAP1, but the exact mechanism is still unknown. It involves, at least partially, the ubiquitination of nucleosome by polycomb complex 1. In case of BAP1 loss in particular cell types, the apoptosis is enhanced by ubiquitin ligase RING1B (part of polycomb complex 1) through the ubiquitination of H2A on prosurvival genes (*Bcl2* and *Mcl1*), that results in their repression. [13]

The present functional study showed that BAP1 increased cell proliferation and migration. It also helped DNA repair in cells after exposure to IR.

Conclusion:

In light of the present results, it could be concluded that mutant BAP1 is localized in a different way in the same type of uveal melanomas cells (nuclear or cytoplasmic or absent), and this may be related to different clinical signs. Therefore, further studies investigating the localization of BAP1 in relation to clinical signs and tumor prognosis are needed.

The results of this work also confirmed that BAP1 plays a role in various cell processes that are implicated in cancer e.g. cell proliferation, migration, and DNA repair. Further investigations are required to find out whether BAP1 mutations in cancer cells affect its functions or not.

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