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Activation of BTAK expression in primary ovarian surface epithelial cells of prophylactic ovaries

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The ovarian epithelial cells carrying heterozygous *BRCA1* or *BRCA2* mutation (we called *BRCA1/2* mutation) are known to predispose to the development of ovarian cancer; however, the molecular basis of such predisposition is largely unknown. We hypothesize that BTAK may be a potential target for heterozygous *BRCA1/2* mutation. We sought to determine the relationship between the status of *BRCA1/2* heterozygous mutation and BTAK expression in prophylactically removed ovaries as compared with normal ovaries and ovarian cancer controls. Western blot analysis of BTAK was performed in a primary cell culture carrying heterozygous *BRCA1* mutation and three normal ovarian surface epithelial cell cultures. Immunohistochemical analysis of BTAK expression was also performed by image analysis in ovaries of 21 patients with known *BRCA1/2* mutation or very strong family history of breast/ovarian cancer that underwent prophylactic oophorectomy, 38 normal ovaries from patients without any known mutation, and 194 ovarian carcinomas. The BTAK expression was significantly increased in primary culture carrying a heterozygous *BRCA1* mutation as compared to those with no known *BRCA1/2* mutation. Immunohistochemical staining of BTAK showed increased expression in ovarian epithelial cells carrying *BRCA1/2* mutation or strong breast/ovarian family history compared with normal ovaries ($P<0.001$). Higher BTAK expression was found in ovarian cancer cells compared to ovaries without cancer but with known *BRCA1/2* mutation or strong family history ($P<0.001$), and expression levels of BTAK and p53 were directly correlated ($r=0.306$; $P<0.001$). Increased expression of BTAK is directly correlated with mutation status of *BRCA1/2* genes, suggesting that mutation in a single allele of either *BRCA1* or *2* may be responsible for the activation of BTAK. This activation may be a key early genetic event in the development of hereditary ovarian cancer.
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BRCA, breast cancer susceptibility gene, plays an important role in maintaining genomic stability and acts as a tumor suppressor. Mutations of *BRCA1* and *BRCA2* genes are associated with increased susceptibility for breast and ovarian cancer. These mutations increase the risk for developing ovarian cancer to 26% (*BRCA1*) and 10% (*BRCA2*) during a woman's lifetime.[1] Since the cloning of both genes more than a decade ago, enormous amount of work has been done to elucidate their functions. It has been shown that both BRCA1 and 2 are involved in multiple cellular pathways.[2,3] *BRCA1* can arrest cell cycle progression by stimulating the transcription of the cyclin-dependent kinase (CDK) inhibitor $p21^{WAF/Cip1}$.[4] *BRCA1* also binds and works cooperatively with *p53 in vivo*;[5] *p53*, in turn, modulates *BRCA1* expression.[6] *BRCA*-deficient cells display spontaneous chromosomal abnormalities, defective G_2/M transit, centrosome amplification, and defects in both homologous DNA recombination and transcription-coupled base-excision repair of oxidative DNA damage.[7,8] Cells with defective *BRCA1* are hypersensitive to DNA-damaging agents, are slower

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to repair double-stranded DNA breaks, and show impairment in transcription-coupled repair.[7] *BRCA1* can also promote homologous recombination, and has been to shown to interact with other proteins required for DNA end-joining, nucleotide mismatch repair, DNA replication, homologous recombination repair, and signal transduction in response to damage. BRCA2 repairs the double-strand break during the S phase of the cell cycle and also participates in cytokinesis. The abnormalities of chromosome number seen in BRCA2-deficient cells are a direct consequence of BRCA2 dysfunction. Moreover, BRCA2 role in cytokinesis provides a mechanism for the generation of polyploidy.[9] All of this suggests that *BRCA* may have multiple functions in the maintenance of genetic stability and cell cycle progression. However, one unsolved puzzle is how the heterozygous *BRCA* mutation in histologically normal ovarian surface epithelial cells predisposes to cancer development in the ovary, particularly whether or not these cells exhibit the haplotype insufficiency.[10–12]

One common characteristic of human cancers is genetic instability, which expresses in numerical and structural abnormalities of the chromosomes. During cell division, centrosomes play an important role in the equal separation of the chromosomes. *BTAK*, encoding a centrosome-associated kinase, regulates the mitotic progression.[13,14] This kinase, with synonyms such as Aurora-2, Aurora-A, ARK1, STK15, is involved in the chromosomal segregation and centrosome translocation to the mitotic spindle in the early mitotic phase. Overexpression of BTAK also results in defective spindle assembly checkpoint, allowing cells with abnormal chromosomal separation to enter anaphase, leading to aneuploidy.[15] Recent studies have demonstrated that activation of BTAK is required for mitotic entry, centrosome maturation and separation, and G_2 to M transition.[16,17] Human BTAK is amplified and overexpressed in various carcinomas such as breast, colorectal, gastric, pancreatic tumors as well as ovarian, bladder, and esophageal cancers.[18–21] These studies indicate that BTAK may play a key role in the pathogenesis of human cancers through the maintenance of genetic stability.

Recent studies have suggested that *BTAK* physically binds and phosphorylates *BRCA* impairing the regulation of the G2/M transition.[22] Studies from *BRCA1* knockout mice have demonstrated that *BRCA* is essential in centrosome localization and duplication, which suggests *BRCA* plays an important role in mitosis,[23,24] thus *BRCA1* and *BTAK* involve similar cellular processes. We hypothesize that *BRCA1* and *BTAK* interactions play a critical role in ovarian tumorigenesis and such interaction occurs at a very early stage and even in primary cells carrying a heterozygous mutation. To test this hypothesis, we examined the expression of BTAK in ovaries from 21 patients with known *BRCA1/2* mutation, 38 normal ovaries, and 194 ovarian cancers with the purpose of establishing epidemiological evidence that BTAK may be one of early targets activated by *BRCA* heterozygous mutation.

Materials and methods

Western Blot Analysis

The primary human ovarian epithelial cells OSE72, OSE103, OSE137, and OSE76 were cultured with complete medium containing 15% fetal bovine serum (FBS) described previously.[25] Cells (OSE72, OSE103, OSE137, and OSE76) were washed twice with phosphate-buffered saline (PBS) and homogenized in lysis buffer (150 mM NaCl, 50 mM HEPES (pH 7.2), 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1% Tween 20, 0.1 mM phenylmethylsulfonylfluoride, 2.5 μg/ml leupeptin, 0.1 mM sodium pyrthovanadate) (Sigma Chemicals, St Louis, MO, USA) at 4°C for 30 min. Lysates were then spun at 10 000 *g* for 10 min at 4°C. The resulting supernatant was transferred to a clean tube and the total protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories). The samples were then boiled in polyacrylamide gel sample buffer containing SDS (SDS sample buffer) for 5 min. Equal amounts (50 μg) of total protein was separated by 12% SDS-PAGE gels and transferred to Hybond-C nitrocellulose membranes (Amersham Life Science) by electroblotting. The membranes were blocked in 5% solution (TBS-T containing skim milk) overnight and then sequentially incubated with the affinity-purified rabbit polyclonal BTAK antibody (Gene Tex; 1:1000, San Antonio, TX, USA) as primary antibody and horseradish-peroxidase-conjugated goat anti-rabbit IgG as a secondary antibody (NA 934V, Amersham Pharmacia) and visualized by using the electrochemiluminescence (ECL) (Amersham Pharmacia Biotech) detection system. To confirm equivalent loading of total protein in all lanes, the membranes were reprobed with β-actin antibody.

Tissue Sample

Ovaries from 21 patients that underwent prophylactic oophorectomy (University of Colorado Health Sciences Center) for known *BRCA1* (5 cases) or *BRCA2* mutation (6 cases) or strong personal and family history of breast/ovarian cancer (10 cases) were included in this study. Pathology of oophorectomy specimen was reviewed by single pathologist (MS). In addition, 38 normal ovaries and 194 cases of ovarian high-grade serous carcinomas were also studied (University of Texas MD Anderson Cancer Center). Normal ovaries and ovarian carcinoma cases were selected from cases that had undergone initial surgery at the University of Texas MD Anderson Cancer Center between 1990 and 2000. The use of tissue followed IRB-approved protocol. Tissue microarray construction was

performed as previously described.[26] Cases in which no tumor was found or no epithelial cells were present were excluded from the final data analysis.

Immunohistochemical Analysis

The tissue slides were subjected to immunohistochemical staining as follows. After initial deparaffinization, endogenous peroxidase activity was blocked by using 0.3% hydrogen peroxide. Deparaffinized sections were microwaved in 10 mM citrate buffer (pH 6.0) to unmask the epitopes. The slides were then incubated against BTAK (Gene Tex; 1:100, San Antonio, TX, USA) and p53 (Santa Cruz; 1:1000, Santa Cruz, CA, USA) for 1 h at ambient temperature. Next, the slides were incubated with biotin-labeled secondary antibody for 20 min, and finally with a 1:40 solution of streptavidin:peroxidase for 20 min. Tissues were then stained for 3 min with freshly prepared 0.05% 3′, 3-diaminobenzidine tetrahydrochloride in 0.05 M Tris buffer at pH 7.6 containing 0.024% H_2O_2 and then counterstained with hematoxylin, dehydrated, and mounted. All of the dilutions of antibody, biotin-labeled secondary antibody, and streptavidin-peroxidase were made in PBS (pH 7.4) containing 1% bovine serum albumin.

Quantitation of Immunohistochemistry

The BTAK intensity of staining was analyzed by computerized image analysis (Ariol SL-50, Applied imaging, San Jose, CA, USA). Quatitation is carried out by measuring all the pixels in a digitalized image and calculating the average. A black pixel has a value of 0 and a white pixel a value of 255. Therefore, a dark staining will have a low value (close to 0) and a light staining a higher value (close to 255). The mean relative optical density was expressed as arbitrary units of intensity and used for analysis. For statistical purposes, optical density values were grouped in a 4-score grading system as the mean optical density $\pm$ s.d. (177.46 ± 15.10). Absence of staining was defined as negative and given a score of 0 (the optical density ≥ 190), weak expression a score of (optical density ≥ 180 and <190), moderate expression a score of 2 (the optical density ≥ 170 and <180), and strong expression a score of 3 (optical density <170). Evaluation of the average expression for p53 expression was performed visually by two pathologists (ZZ and DGR), independently in a blinded manner as follows: 0, less than 10% nuclear staining; 1, more than 10%, less than 25% nuclear staining; 2, more than 25%, less than 50% nuclear staining; 3, more than 50% nuclear staining, discrepancy was resolved by third pathologist (JL).

Statistical Analysis

Differences in proportions were evaluated by the χ^2 analyses. Kruskal–Wallis and Mann–Whitney test were used to compare multiple independent samples containing normal ovaries, mutation ovaries, and ovarian carcinomas. The relationship between expression of BTAK and p53 was analyzed with Spearman Rank Order Correlation test. The basic descriptive statistical analysis and tables were created using the Statistica software package, version 6.0 (Statsoft, Tulsa, OK, USA). Results were considered statistically significant at the $P<0.05$ level.

Results

Expression of BTAK in Primary Ovarian Epithelial Cell Cultures with or without *BRCA1* Heterozygous Mutation

BTAK expression was examined by western blot analysis in four primary ovarian epithelial cell cultures, three of which without known *BRCA* mutation (OSE72, OSE103, and OSE137) and one with a known *BRCA1* mutation (OSE76). A specific band of BTAK protein (46 kDa) was detected strongly in OSE76 cell line by western blot, but no clear bands were found in OSE72, OSE103, and OSE137 (Figure 1), suggesting that BTAK expression was increased in primary ovarian epithelial cells carrying a *BRCA1* mutation.

Comparison of BTAK and P53 Expression in Normal Ovaries, Ovaries with *BRCA* Mutations or Strong Family History of Breast/Ovarian Cancer and Ovarian Carcinomas

Next, we performed immunohistochemical staining of morphologically normal ovaries in a cohort of 21 patients who had prophylactic surgery and compared these with 38 normal ovaries and 194 ovarian cancer controls. Results are shown in Table 1. The expression of BTAK showed 1+ positivity in eight of 21 cases, 2+ positivity in seven of 21 cases, and 3+ positivity in one of 21 cases in ovaries carrying a heterozygous *BRCA* mutation as compared with 38 normal control ovaries, which showed 1+ positivity in seven of 38 cases (18%), 2+ positivity in two

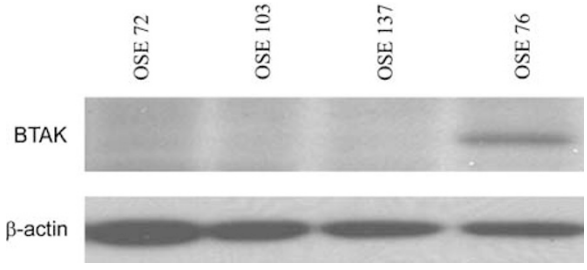


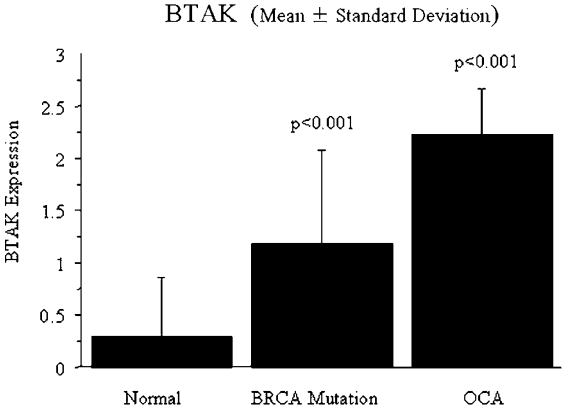
Figure 1 Western blot analyses of OSE76 showed overexpression of BTAK, compared with OSE72, OSE103, and OSE137, which do not show BTAK expression. Blots were stripped and reprobed with a human β-actin probe to confirm equal loading. In total, 50 μg of total protein was loaded in each lane.

Table 1 Expression of biomarkers in ovarian tissue samples

Biomarker by tissue type	*Expression level,* n *(%)*				
	0	*1*	*2*	*3*	P-*value*
BTAK					
Normal ovaries (38)	29 (76)	7 (18)	2 (5)	0 (0)	$<0.001^a$
Ovaries with BRCA mutations or strong family history (21)	5 (24)	8 (38)	7 (33)	1 (5)	$<0.001^b$
Carcinoma (194)	0 (0)	2 (1)	146 (75)	46 (24)	$<0.001^c$
p53					
Normal ovaries (38)	34 (89)	2 (5)	1 (3)	1 (3)	$<0.001^a$
Ovaries with BRCA mutations or strong family history (21)	14 (67)	0 (0)	4 (19)	3 (14)	0.023^b
Carcinoma (194)	55 (28)	23 (12)	20 (10)	96 (50)	$<0.001^c$

Reference: *P*-values reflect differences in proportions between [a]normal ovarian tissue *vs* ovarian carcinomas, [b]normal ovarian tissue *vs* ovarian tissue carrying BRCA heterozygous mutations or prophylactic oophorectomy ovaries, and [c]ovarian tissue carrying BRCA heterozygous mutations or prophylactic oophorectomy ovaries *vs* ovarian carcinomas. Percentages represents positive or negative case number ratio.

of 38 (5%), and no 3+ positivity in any of the normal ovaries ($P<0.001$; Mann–Whitney test), while ovarian cancer showed further increase in BTAK expression in all 194 cases as compared with *BRCA* heterozygous ovaries ($P<0.001$; Mann–Whitney test). The expression of p53 showed moderate to strong positivity in two of 38 cases (6%) in normal ovary, weakly increased expression in seven of 21 cases (33%) with known *BRCA* mutation or strong family history. p53 is highly expressed in 116 of 194 (60%) of ovarian carcinomas. Using Spearman correlation rank order test, we observed BTAK was correlated with p53 ($r=0.306$, $P<0.001$). The summary of BTAK expression is shown in Figure 2. Representative pictures of immunohistochemical staining for BTAK and p53 in normal ovary, prophylactic ovary and ovarian cancer are shown in Figure 3.



	Count	Mean	Std. Dev.
Normal	38	0.289	0.565
BRCA Mutation	21	1.190	0.873
OCA	194	2.227	0.444

Figure 2 This chart illustrates the results from a quantitative analysis of BTAK expression in ovarian surface epithelial cells of normal ovary, ovaries carrying heterozygous *BRCA* mutation or with strong family history of breast and ovarian cancer, and ovarian carcinoma (OCA). (Kruskal–Wallis test; $P<0.05$).

Discussion

One of major unresolved issues in the field is whether *BRCA* heterozygous mutation carriers exhibit different phenotype from non-mutational carriers. While several studies have failed to detect such difference at the histopathological level,[11,27–30] others have detected such differences between these two different groups.[10,12,31–37] In addition, human ovarian-surface epithelial (HOSE) cells from patients with a family history of breast or ovarian cancer show increased CA-125 expression[34] and more stable expression of the Met receptor for hepatocyte growth factor (HGF) than do HOSE cells from patients without such a history.[38] Transformation of HOSE cells with the SV40 T/t antigen led to increased telomere instability and reduced growth potential in cells from patients with a family history of breast or ovarian cancer, indicating that those cells were closed to replicative senescence.[39] Furthermore, He[40] found that several genes involved in protein synthesis are upregulated in ovarian surface epithelial cells derived from prophylactic oophorectomies but not in those without *BRCA* mutations. All of these findings suggest that ovarian epithelial cells with heterozygous *BRCA1* or *BRCA2* germline mutations are biologically different from cells without such germline mutations, either through haplo-insufficiency or through predisposition to a loss of the second wild-type allele of the *BRCA1* gene. However, the differences between these two groups are relatively subtle as reflected in the existing conflicting literatures on this topic. The results described here support a latter view: HOSE cells carrying heterozygous mutations are different from those without mutations, and BTAK is a potential target that is activated by a heterozygous *BRCA1* or *BRCA2* mutation in morphologically normal ovarian surface epithelial cells.

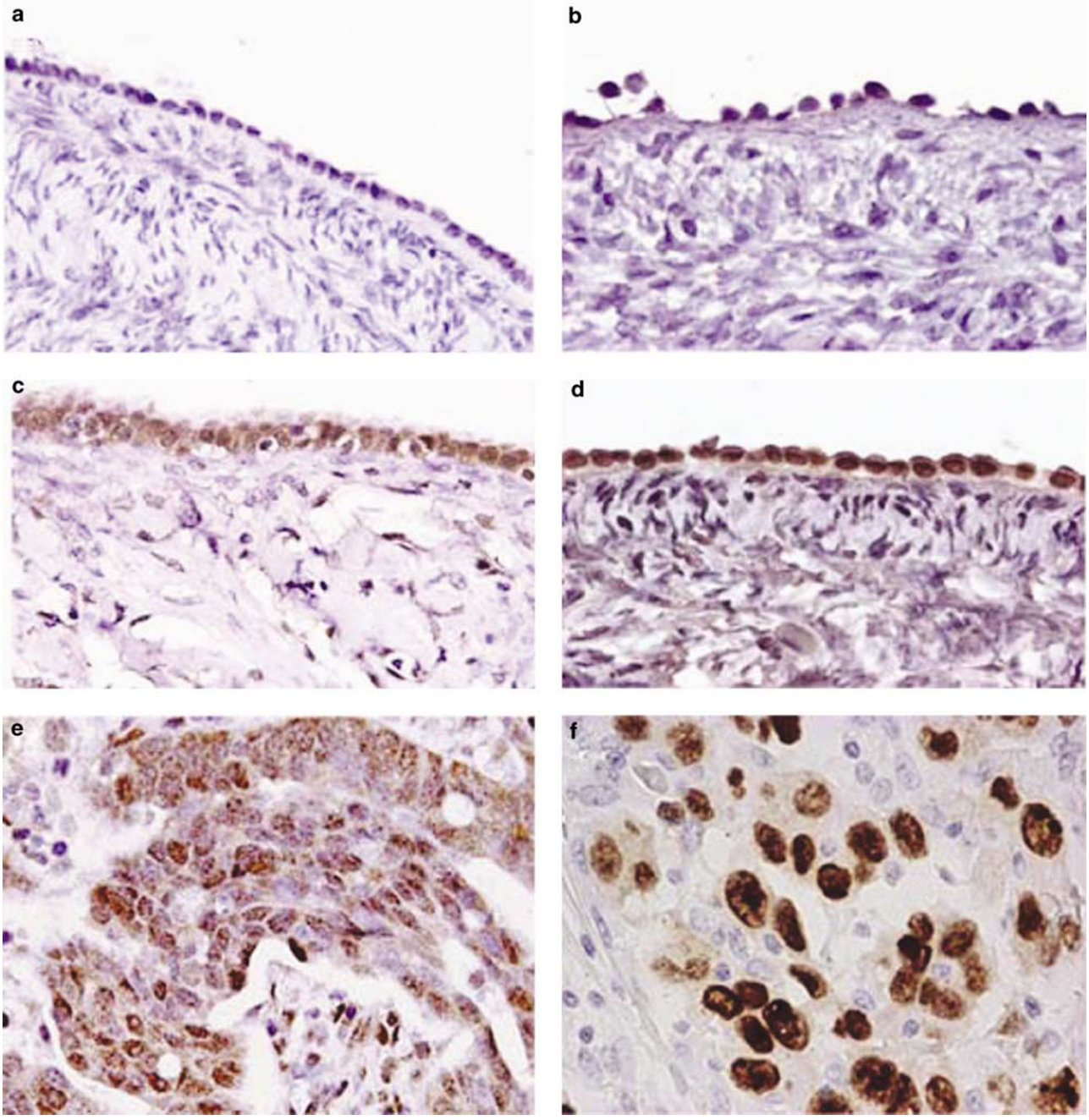


Figure 3 Immunohistochemical staining for BTAK and p53 protein expression. (**a**) No expression of BTAK was found in normal ovarian surface epithelial cells. (**b**) No expression of p53 was found in normal ovarian surface epithelial cells. (**c**) Cytoplasmic expression of BTAK was found in the ovarian epithelial cells carrying *BRCA* mutation. (**d**) Nuclear staining of p53 was found in the ovarian epithelial cells carrying *BRCA* mutation. (**e**) Strong cytoplasmic staining of BTAK was found in the OCA. (**f**) Strong nuclear staining of p53 was found in the OCA. Sections were counterstained with hematoxylin. Original magnification $\times 400$.

Human *BTAK* gene is located in the 20q13 chromosome region and is involved in the G_2–M checkpoint and mitosis commitment.[41] It is amplified and overexpressed in several different types of malignant tumors including ovarian carcinomas.[42,43] Recent studies reported that BRCA is also localized in the centrosome [23,44] and BTAK and BRCA1 form a complex.[22] Ouchi *et al*[22] showed recently that BTAK physically binds and phosphorylates BRCA1, and the phosphorylation is correlated with impaired function of BRCA1 in regulating G2/M transition. This suggests a link between BTAK expression and impaired BRCA1 function in genetic instability and tumorigenesis. Our results suggest that BTAK is overexpressed in prophylactic ovaries with known *BRCA* mutation. Biochemical evidence showed that BTAK and BRCA form a complex *in vivo* and BRCA1 is the substrate of BTAK. Mutation in one of two alleles of *BRCA* may decrease the physiological substrate for BTAK and lead to further increase in

BTAK expression.[45] Such increased BTAK activity may lead to genetic instability and *p53* inactivation, which can lead to initiation of transformation of ovarian epithelial cells. Our results are consistent with several recent reports, which show that the activation and overexpression of BTAK is more frequent in early stage/low-grade ovarian tumors [46] and is proved to be an early event in tumorigenesis in a rat mammary carcinogenesis model.[47]

Another interesting finding is that BTAK expression by immunohistochemistry was correlated with p53 expression. *p53* is a well-known tumor suppressor gene that is mutated in nearly 50% of all human tumors.[48] Previous studies have showed that overexpression of BTAK phosphorylates p53, leading to its degradation and decreasing p53 level, then inducing oncogenic transformation.[49] Their results suggested that expression of BTAK induces tumorigenesis through degradation of p53 and BTAK. Phosphorylation of p53 is associated with BTAK-regulated cell cycle progression, cell survival, and transformation. These data indicate that p53 may be a physiological substrate of BTAK that may exert its function through phosphorylation of p53. Our finding of high p53 expression level, most likely indicative of mutant forms, has been shown to be associated with high BTAK expression. However, the underlying mechanisms of how the interaction of BRCA, p53, and BTAK regulate the initiation of ovarian tumorigenesis remain to be tested in future experiments.

In summary, we provide strong evidence that BTAK is overexpressed in the ovaries of women with BRCA mutation or strong family history of breast and ovarian cancer compared with normal ovaries from women who lack *BRCA* mutation. BTAK expression is associated with elevated mutant p53. As several inhibitors for BTAK are at various stage of clinical trials,[50,51] BTAK may offer a novel target for chemoprevention to decrease ovarian cancer risk and save patient from a drastic surgical procedure, prophylactic oophorectomy.

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