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Quantitative and correlation analysis of the DNA methylation and expression of DAPK in breast cancer

Background. Death associated protein kinase 1 (DAPK) is an important tumor suppressor kinase involved in the regulation of multiple cellular activities such as apoptosis and autophagy. DNA methylation of DAPK gene was found in various types of cancers and often correlated with the clinicopathological characteristics. However, the mRNA and protein expression of DAPK in the same sample was rarely measured. Thus it was unclear if the correlation between DAPK gene methylation and clinicopathological parameters was due to the loss of DAPK expression.

Methods. In this study, the DNA methylation rate, mRNA and protein expression of DAPK was quantitatively detected in 15 pairs of breast cancer patient samples including tumor (T) and adjacent non-tumor (N) tissues. **Results.** The correlation between DNA methylation rate and mRNA expression, together with the correlation between mRNA and protein expression, was calculated. No correlation was observed between any levels using either the measurement value of each sample or the T/N ratio of each pair.

Discussion. These data suggested the DNA methylation status of DAPK did not correlate well with its mRNA or protein expression. Extra caution is needed when interpreting the DNA methylation data of DAPK gene in clinical studies.

Quantitative and correlation analysis of the DNA methylation and expression of DAPK in breast cancer

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Abstract

Background. Death associated protein kinase 1 (DAPK) is an important tumor suppressor kinase involved in the regulation of multiple cellular activities such as apoptosis and autophagy. DNA methylation of DAPK gene was found in various types of cancers and often correlated with the clinicopathological characteristics. However, the mRNA and protein expression of DAPK in the same sample was rarely measured. Thus it was unclear if the correlation between DAPK gene methylation and clinicopathological parameters was due to the loss of DAPK expression.

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Key words: DAPK; breast cancer; DNA methylation; Correlation;

Introduction

Death associated protein kinase 1 (DAPK) is an important tumor suppressor kinase that participates in apoptosis, autophagy, cell migration and so on (Lin, Hupp, & Stevens, 2010; Schneider-Stock, 2014; Shiloh, Bialik, & Kimchi, 2014). The expression of DAPK is often lost in various types of tumor due to DNA hypermethylation (Benderska & Schneider-Stock, 2014). The DNA methylation of DAPK was found to be correlated with multiple clinicopathological characteristics in many cancer types (Benderska & Schneider-Stock, 2014). However in the over 100 studies on DAPK methylation in cancer in the past two decades, only few of them detected the mRNA or protein levels of DAPK in the same patient samples (2014). It was unclear whether the hypermethylation of DAPK gene actually correlate with down-regulation of DAPK mRNA or protein expression. Therefore, it is important to investigate the DNA methylation and expression of DAPK in the same patient samples for a more accurate mechanistic explanation of the clinical significance of DAPK DNA methylation.

The DNA methylation of DAPK in breast cancer was examined in several studies (Lehmann et al., 2002; Van der Auwera et al., 2009; Van der Auwera et al., 2010). However, the protein expression of the matched samples was not tested in these studies. Hence it was not yet clear if the DNA methylation status of DAPK in breast cancer correlated with its mRNA or protein expression. Since 1990s, the incidence of breast cancer in China has increased dramatically at a speed twice as fast as the rest of the world. At present, breast cancer is the most common female cancer and ranked sixth for cancer associated death in China (Fan et al., 2014). It will be interesting to compare the methylation status and expression levels of DAPK in a Chinese patient cohort.

In this study, we employed a quantitative method to measure the DNA methylation of DAPK gene together with the expression DAPK mRNA and protein in 15 pairs of breast cancer patient samples. The correlation between the DAPK DNA methylation rate, mRNA expression and protein levels in these samples were investigated.

Materials and Methods

Clinical samples

The 15 pairs of breast cancer patient samples were obtained from patients with invasive ductal carcinoma at the first affiliated hospital of Fujian Medical University. Written consent was obtained for all patients.

DNA extraction and Bisulfite Modification

Genomic DNA from frozen specimens with the weight about 30~60mg was extracted by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation, dissolved in 50~100 ml of distilled water, and stored at -20°C until usage. About 1 µg of genomic DNA was subject to sodium bisulfite modification to convert unmethylated cytosines to uracils. All the procedures were performed according to the CpGenome™ Universal DNA Modification Kit (Millipore, Massachusetts, USA) before the samples were subject to polymerase chain reaction (PCR).

Construction of plasmid “ pUC57-Methyl ”

The sequences of the PCR products for both the M and U primers (Figure 1B) are cloned into the plasmid pUC57 and named pUC57-methyl (Sangon Biotech, Shanghai, China).

Methylation Specific PCR (MSP)

In a typical experiment, 5µl modified DNA or 1µl of synthesized plasmid was used as the template in a total volume of 25µl. The MSP solution contained 1×EX *Taq* buffer, 2.5 mM dNTP 2µl, 10 µM PCR primers 0.5µl, and 0.125µl EX *Taq* HS DNA polymerase (TaKaRa). PCR conditions are: 95°C for 5 min, 35 cycles at 95°C for 15 s, 55°C for 15 s, 72°C for 15 s and a 5 min extension was allowed at 72°C.

RNA extraction and detection

The mRNA from patient tissue was extracted using the Easstep™ Total RNA Extraction Kit (Promega, Beijing, China) and reverse transcribed using the GoScript™ Reverse transcription system (Promega, Madison, USA) according to the manufacturers' instruction. The Real-Time PCR primers for *DAPK* (NCBI Reference Sequence: NG_029883.1) and Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; NCBI Reference Sequence: NG_007073.2) were listed on Table 1. PCR reaction conditions were as follows: 95°C for 2

min, 40 cycles at 95°C for 15 s and 60°C for 1 min. The cDNA from HEK293T cells were used as positive control and standard curve.

Protein extraction and Western blotting

The total protein from frozen specimens with the weight about 30~60mg was extracted using 100~200µl of radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) containing protease inhibitors (Roche, Basel, Switzerland). Protein concentration was determined using BioTek Dc Protein Assay (BioTek, Hercules, CA, USA). Equal amount of lysates (60-90µg) were subject to SDS-PAGE. The primary anti-DAPK antibody is from LSBio (LifeSpan BioSciences, Inc). The horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies were from Sigma-Aldrich (St. Louis, MO).

Results

Quantitative Measurement of DAPK Promoter Hypermethylation

The first report on DAPK promoter hypermethylation was published in 1999 by Katzenellenbogen et al (Katzenellenbogen, Baylin, & Herman, 1999). Since then, most DNA methylation studies on DAPK gene was carried out using one set of MSP primers (Table 1) (Huang et al., 2014; Katzenellenbogen, Baylin, & Herman, 1999). Therefore, we initially carried out the same MSP approach in three randomly picked pairs of breast cancer samples and successfully detected the methylated (M) and un-methylated (U) bands (Figure 1A). However, it is difficult to quantitatively measure the methylation rate due to the differential cell composition in each sample and the binding affinity of the M and U primers. Therefore, we designed pUC57-methyl construct containing the PCR products for both the M and U primers (Figure 1B) to allow quantitative measurement. The PCR reaction using pUC57-methyl as the template was clean, confirming the validity of this construct (Figure 1C). Next we measured the methylation rate of 15 pairs of breast cancer patient samples using real-time PCR (Figure 1D). The standard curve was created using the engineered construct. The methylation rate was calculated by comparing the PCR product of M against the total (M+U) for each sample, so that the problem of differential cell composition was avoided.

Lack of Correlation Between DAPK mRNA Expression and DNA methylation

Next the DAPK mRNA expression of these 15 pairs of breast cancer samples were

analyzed using real-time PCR (Figure 2A). Similar to the DNA methylation data (Figure 1D), differential expression was observed across the samples (Figure 2A). In order to investigate whether the DNA methylation status correlate with the mRNA expression, a correlation analysis was carried out. No correlation was observed between DAPK DNA methylation rate and its mRNA expression in the total of 30 samples (Figure 2B). Further, the tumor/normal (T/N) ratio of the 15 samples were used for the correlation analysis. Again no correlation was observed between the T/N ratio of DAPK DNA methylation status and its mRNA expression (Figure 2C).

Lack of Correlation between DAPK protein and mRNA expression

The DAPK protein expression of these 15 pairs of breast cancer samples were then analyzed using western blot (Figure 3A). No correlation between the DAPK protein and mRNA expression in the total of 30 samples was observed (Figure 3B). Moreover, no correlation between the T/N ratio of DAPK protein and mRNA expression of the 15 patient samples was observed either (Figure 3C).

Discussion

In this study, we engineered an artificial construct pUC57-methyl to quantitatively measure the DNA methylation rate of DAPK. Using pUC57-methyl, we can resolve the differential affinity problem between the U and M primers and directly compare the proportion of methylated and unmethylated DAPK gene within the same sample. Thus we can avoid the problem of differential cell composition across the samples and only investigate the changes of the proportion of methylated DAPK gene. However, it was clear that the methylation rate could vary dramatically from one patient to another even in the non-tumor tissues (Figure 1D). One assumption we need for this type of study is that the tumor and non-tumor samples from the same patient have about the same cell composition. It will be ideal if this study can be performed only in the cancer cell subgroup.

As mentioned above, most studies on DAPK DNA methylation used the same set of primers that targets a specific site on DAPK promoter (Katzenellenbogen, Baylin, & Herman, 1999). There is no doubt this site is critical for regulating the transcription of DAPK gene. However, there are multiple CpG islands on DAPK promoter (Benderska & Schneider-Stock,

2014). It is possible some other sites can also participate in the regulation of DAPK mRNA expression. Moreover, there are no reports on the regulatory element such as enhancer for DAPK gene. The individual status of these transcriptional regulators may also influence the expression of DAPK mRNA. Actually it was reported before that DAPK protein expression could still be detected at the presence of DNA methylation in non-small lung cancer (NSCLC), renal cell carcinoma (RCC) and chronic lymphoid leukemia (CLL) (Huang et al., 2014; Toyooka et al., 2003), supporting that more components need to be taken into account when interpreting DAPK DNA methylation data.

The protein expression of DAPK is controlled by both ubiquitin-proteasome (Jin et al., 2002; Lee et al., 2010; Zhang, Nephew, & Gallagher, 2007) and lysosome pathways (Gallagher & Blue, 2014; Lin et al., 2011; Lin et al., 2009; Lin et al., 2008; Stevens et al., 2009). It is not surprising that the mRNA and protein expression of DAPK did not correlate well (Figure 3). But protein expression is the endpoint of gene expression. If the DNA modification or mRNA levels of DAPK cannot correlate with its protein level, extra caution was needed for interpreting the correlation between their correlations with clinicalpathological parameters as they may actually reflect the activity or expression of the regulatory components rather than the actual DAPK protein expression.

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Conflict of interest: The authors claim no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

References

- Benderska N, Schneider-Stock R. (2014). Transcription control of DAPK. *Apoptosis*, 19(2), 298-305
- Fan L, Strasser-Weippl K, Li J J, St Louis J, Finkelstein D M, Yu K D, Chen W Q, Shao Z M, Goss P E. (2014). Breast cancer in China. *Lancet Oncol*, 15(7), e279-289
- Gallagher P J, Blue E K. (2014). Post-translational regulation of the cellular levels of DAPK. *Apoptosis*, 19(2), 306-315
- Huang Y, Chen L, Guo L, Hupp T R, Lin Y. (2014). Evaluating DAPK as a therapeutic target. *Apoptosis*, 19(2), 371-386
- Jin Y, Blue E K, Dixon S, Shao Z, Gallagher P J. (2002). A death-associated protein kinase (DAPK)-interacting protein, DIP-1, is an E3 ubiquitin ligase that promotes tumor necrosis factor-induced apoptosis and regulates the cellular levels of DAPK. *The Journal of biological chemistry*, 277(49), 46980-46986
- Katzenellenbogen R A, Baylin S B, Herman J G. (1999). Hypermethylation of the DAP-kinase CpG island is a common alteration in B-cell malignancies. *Blood*, 93(12), 4347-4353
- Lee Y R, Yuan W C, Ho H C, Chen C H, Shih H M, Chen R H. (2010). The Cullin 3 substrate adaptor KLHL20 mediates DAPK ubiquitination to control interferon responses. *EMBO J*, 29(10), 1748-1761
- Lehmann U, Celikkaya G, Hasemeier B, Langer F, Kreipe H. (2002). Promoter hypermethylation of the death-associated protein kinase gene in breast cancer is associated with the invasive lobular subtype. *Cancer Res*, 62(22), 6634-6638
- Lin Y, Henderson P, Pettersson S, Satsangi J, Hupp T, Stevens C. (2011). Tuberous sclerosis-2 (TSC2) regulates the stability of death-associated protein kinase-1 (DAPK) through a lysosome-dependent degradation pathway. *FEBS J*, 278(2), 354-370
- Lin Y, Hupp T R, Stevens C. (2010). Death-associated protein kinase (DAPK) and signal transduction: additional roles beyond cell death. *FEBS J*, 277(1), 48-57
- Lin Y, Stevens C, Harrison B, Pathuri S, Amin E, Hupp T R. (2009). The alternative splice variant of DAPK-1, s-DAPK-1, induces proteasome-independent DAPK-1 destabilization. *Mol Cell Biochem*, 328(1-2), 101-107
- Lin Y, Stevens C, Hrstka R, Harrison B, Fourtouna A, Pathuri S, Vojtesek B, Hupp T. (2008). An alternative transcript from the death-associated protein kinase 1 locus encoding a small protein selectively mediates membrane blebbing. *FEBS J*, 275(10), 2574-2584
- Schneider-Stock R. (2014). Death-associated kinase (DAPK): a cancer "gene chameleon". *Apoptosis*, 19(2), 285
- Shiloh R, Bialik S, Kimchi A. (2014). The DAPK family: a structure-function analysis. *Apoptosis*, 19(2), 286-297
- Stevens C, Lin Y, Harrison B, Burch L, Ridgway R A, Sansom O, Hupp T. (2009). Peptide combinatorial libraries identify TSC2 as a death-associated protein kinase (DAPK) death domain-binding protein and reveal a stimulatory role for DAPK in mTORC1 signaling. *J Biol Chem*, 284(1), 334-344

Toyooka S, Toyooka K O, Miyajima K, Reddy J L, Toyota M, Sathyanarayana U G, Padar A, Tockman M S, Lam S, Shivapurkar N, Gazdar A F. (2003). Epigenetic down-regulation of death-associated protein kinase in lung cancers. *Clin Cancer Res*, 9(8), 3034-3041

Van Der Auwera I, Bovie C, Svensson C, Limame R, Trinh X B, Van Dam P, Van Laere S J, Van Marck E, Vermeulen P B, Dirix L Y. (2009). Quantitative assessment of DNA hypermethylation in the inflammatory and non-inflammatory breast cancer phenotypes. *Cancer Biol Ther*; 8(23), 2252-2259

Van Der Auwera I, Bovie C, Svensson C, Trinh X B, Limame R, Van Dam P, Van Laere S J, Van Marck E A, Dirix L Y, Vermeulen P B. (2010). Quantitative methylation profiling in tumor and matched morphologically normal tissues from breast cancer patients. *BMC Cancer*, 10, 97

Zhang L, Nephew K P, Gallagher P J. (2007). Regulation of death-associated protein kinase. Stabilization by HSP90 heterocomplexes. *J Biol Chem*, 282(16), 11795-11804

Table 1: Summary of Primer Sequences and Product Size Used in the PCR procedure

Gene name	Sequences (5'-3')	Product Size
DAPK methylated	F: GGATAGTCGGATCGAGTTAACGTC R: CCC TCC CAA ACG CCG A	98 bp
DAPK unmethylated	F: GGAGGATAGTTGGATTGAGTTAATGTT R: CAAATCCCTCCCAAACACCAA	106 bp
DAPK RT PCR	F: GATAGAAATGTCCCAAACCTCG R: TCTTCTTTGGATCCTTGACCAGAA	189 bp
GAPDH RT PCR	F: CGGAGTCAACGGATTTGGTCGTAT R: AGCCTTCTCCATGGTGGTGAAGAC	304 bp

RT PCR, Real-Time PCR; F, forward primer; R, reverse primer.

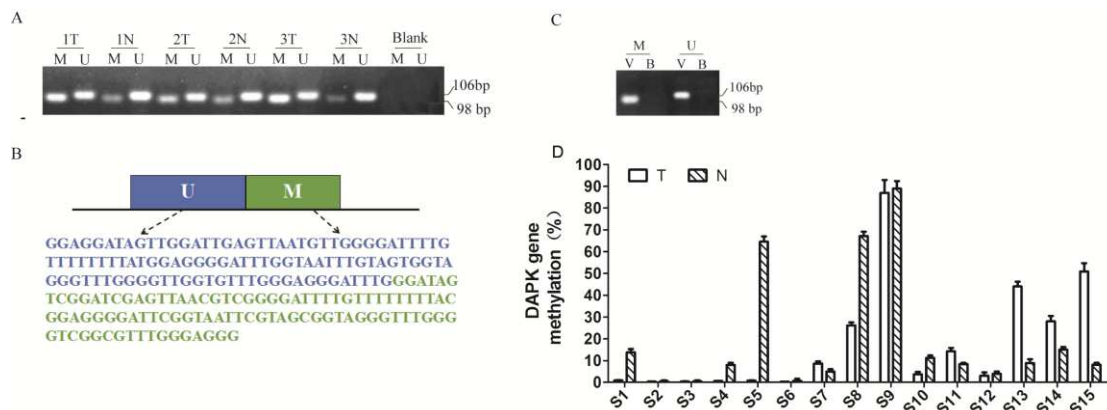


Figure 1

Figure 1: Quantitative detection of *DAPK* promoter methylation in breast cancer samples. **(A)** Detection of *DAPK* promoter methylation in 3 pairs of random breast cancer patient samples. The methylation specific PCR products were resolved in 2% agarose gels. M, methylated *DAPK*; U, unmethylated *DAPK*; Blank, negative control (without DNA); T, the breast cancer tissue; N, the corresponding adjacent normal tissue. **(B)** The PCR product sequences inserted into synthetic plasmid. UM: sequence for unmethylated *DAPK* gene promoter; M: sequence for methylated *DAPK* gene promoter. **(C)** Validation of the engineered construct as PCR template. B, blank; V, vector. **(D)** Quantitative detection of *DAPK* promoter methylation in breast cancer samples using real-time PCR and. T, breast cancer tumor tissue; N, the corresponding adjacent normal tissue; S1~S15, patient codes.

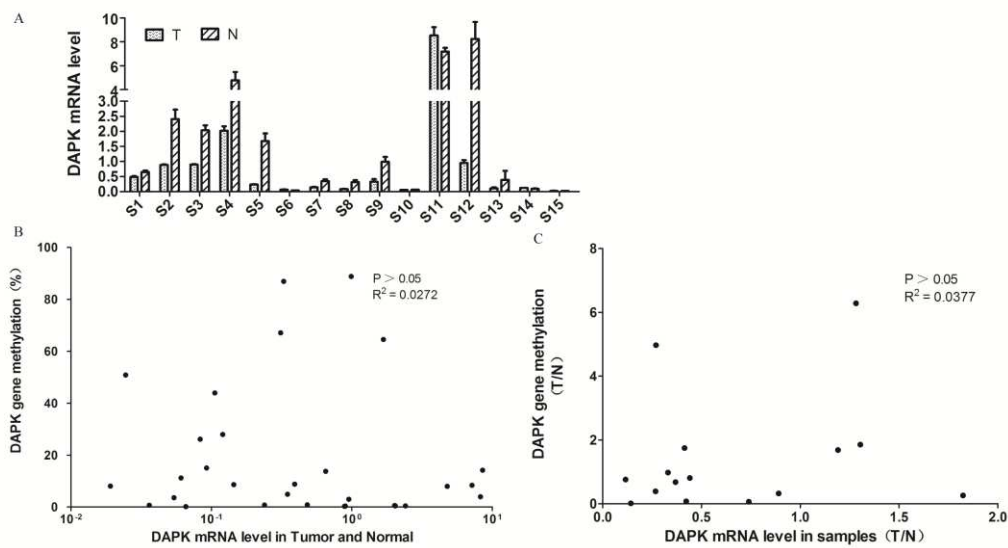


Figure 2

Figure 2: Analysis of the correlation between *DAPK* mRNA and promoter methylation in breast cancer samples. (A) The mRNA expression of *DAPK* was detected using Real-Time PCR. S1~S15, patient codes. (B) Correlation analysis of *DAPK* mRNA expression and *DAPK* promoter methylation rate in breast cancer samples. (C) Correlation analysis of the T/N ratio of *DAPK* mRNA expression and *DAPK* promoter methylation rate in breast cancer samples.

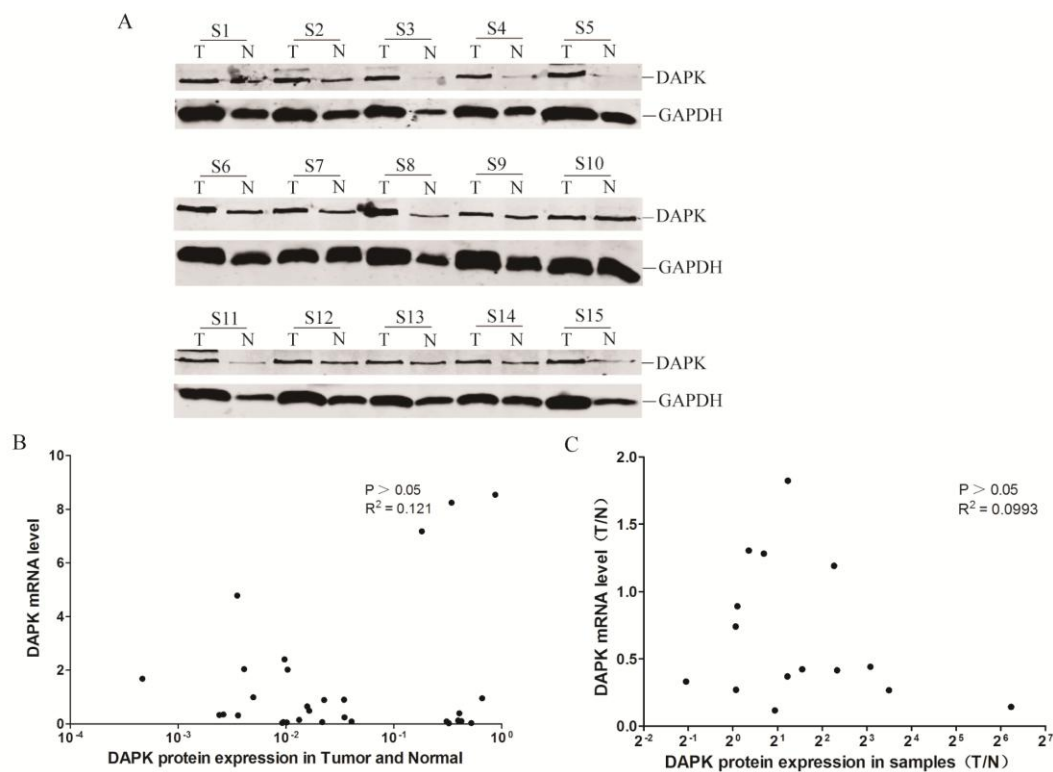


Figure 3

Figure 3: Analysis of the correlation between DAPK mRNA and protein expression in breast cancer samples. **(A)** The protein expression of DAPK was detected using western blot. T, the breast cancer tissue; N, the corresponding adjacent normal tissue; S1~S15, patient codes. **(B)** Correlation analysis of *DAPK* mRNA expression and DAPK protein expression in breast cancer samples. **(C)** Correlation analysis of the T/N ratio of *DAPK* mRNA expression and DAPK protein expression in breast cancer samples.