Expression of *STK11* gene and its promoter activity in MCF control and cancer cells

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Abstract Serine/threonine kinase gene (*STK11*) is identified as tumor suppressor gene whose mutation can lead to Peutz–Jeghers syndrome (PJS). *STK11* is emerging as a multifunctional protein, it activates 14 different AMP-activated protein kinase (AMPK) family members, important in the regulation of cell polarity, cell cycle arrest, energy and hemostasis. Present study was designed to evaluate *STK11* mRNA expression in MCF-7 cancer and MCF-10 normal breast cells lines. mRNA expression was studied by real-time PCR. Further, human *STK11* promoter construct was fused to a luciferase reporter and transfected into both MCF-7 and MCF-10 cells to identify the promoter activity in these cells. *STK11* mRNA was found significantly higher in MCF-7 compared to MCF-10 cells (p value < 0.0005) indicating its role in the onset of breast cancer. Interestingly, it was found that the promoter activity of *STK11* gene in MCF-7 cells was also significantly higher when compared to MCF-10 cells (p value < 0.005). Positive correlation was observed in promoter activity and gene expression ($p = 0.048$, $r^2 = 0.587$). This study for the first time relates the altered *STK11* gene expression in breast cancer cells with altered promoter activity. The present finding may shed light on the new therapeutic approaches against breast cancer by targeting gene or its promoter.

Keywords Serine–threonine kinase 11 · Breast cancer · Expression · Reporter assay · Tumor suppressor

Introduction

Breast cancer is the most common cancer in women with approximately 1.15 million new cases diagnosed annually and increase in death rate with passing years becoming a significant problem worldwide. *STK11* protein, also known as LKB1, is one of the protein kinase that encodes from *STK11* gene and acts as a tumor suppressor. *STK11* was discovered as a mutant gene in patients with Peutz–Jeghers syndrome (PJS) (Peutz 1921). The risk of developing breast cancer in PJS patients is 8% at the age of 40 and reaches 45% by the age of 70 (Hearle et al. 2006). Also mutations in *STK11* gene have been found in patients of breast cancer without PJS. Furthermore, loss of *STK11* expression leads to papillary breast carcinoma (Esteller et al. 2000). This shows that *STK11* gene is an important candidate to explore in breast cancer studies.

The *STK11* protein plays many roles in cell biology at nuclear and cytoplasmic localization. It is a master kinase that regulates diverse cellular processes, including cell cycle arrest, p53-mediated apoptosis, cell polarity and energy metabolism by activating different kinases, the important ones being AMPK (Baas et al. 2004). Interestingly, *STK11* can play an oncogenic role rather than tumor suppressor, which confers a survival advantage under special circumstances, contributing to cancer cell evolution and the rise of progressive cell populations. This controversial expression may be correlated to the different processes of tumorigenesis. It was previously reported that *STK11* acts as a coactivator in estrogen receptor (ER α) signaling, the enhanced expression of which has been

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associated with increased breast cancer risk (Nath-Sain and Marignani 2009). Furthermore, Akt phosphorylation of pro-apoptotic proteins which is supported by STK11 leads to suppress apoptosis in tumor cells (Zhong et al. 2008). Although, there is some information in the molecular roles of *STK11* in controlling cellular processes, but little is known about its transcription regulation. Promoters carry the central regulatory information of genes, therefore, their correct annotation and characterization is vital to understand gene function. *STK11* promoter has distinct cis-regulatory elements, including three CCAAT boxes and GC-box that critically affect *STK11* gene expression. These cis-acting elements are able to bind to transcription factors such as specificity protein 1 (Sp1), nuclear transcription factor Y (NF-Y) and the two ubiquitous transcription factors involved in the regulation of various other genes (Mantovani 1999; Suske 1999). Also, FOXO3 and FOXO4 activate *STK11* gene transcription through interaction with recognition site GTAAACAA (Furuyama et al. 2000). The present study aimed to examine the differential expression of *STK11* gene by real-time PCR and its relation to the differential promoter activity in cancerous MCF-7 cell lines and normal breast MCF-10.

Material and method

Reverse transcription (RT) and real time PCR

RT-PCR was conducted to quantify the expression level of *STK11* gene. Trizol reagent (Sigma) was used to extract total RNA from MCF-7 and MCF-10 cells, cDNA was prepared and reverse-transcription was performed using iScript™ Select cDNA Synthesis Kit (BioRad) with Oligo (dT) primers as directed by the manufacturer. Briefly, 2 µg total RNA, 2 µl of Oligo (dT) primers mix, 4 µl 5X iScript select reaction mix and 1 µl of iScript Reverse transcriptase, made up to 20 µl with nuclease-free water was incubated at 45 °C for 2 h. The reverse transcriptase was inactivated at 85 °C for 5 min and cDNA was stored at –20 °C.

Quantification of human *STK11* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript was performed on the LC-480 system (Roche) in triplicate using primers (STK11-F, 5' GCCGGGACTGACGTGTAGA 3' STK11-R, 5' CCCAAAAGGAAGGGAAAAACC 3', GAPDH-F, 5' ACAGTCAGCCGCATCTTCTT 3' and GAPDH-R, 5' TTGATTTTGGAGGGATCTCG 3'. GAPDH was used as the internal control to normalize template loading quantity. Each reaction contained 5 µl cDNA, 10 µl Master Mix 2X concentration, 3 µl PCR grade water and 2 µl primer (5 µM), in a final volume of 20 µl.

Cycling conditions were one cycle at 95 °C for 5 min, followed by 45 cycles of 95 °C for 20 s, 60 °C for 15 s, and 72 °C for 20 s and melt peak determination. Relative mRNA levels were calculated and results were presented as fold relative to indicated controls.

Cloning

The *STK11* promoter reporter construct was generated by amplifying a 3995 bp fragment of the *STK11* promoter using primers STK11prom-XhoI-F 5' CGGGAATCTC GAGTTGGAAATTCAAGTG TGTAGGGCA 3' and STK11prom-HindIII-R 5' AAAGCGCAAGCTTCAACAA AAACCCCA AAAGGA 3' resulting in amplification of a product containing XhoI and HindIII restriction enzyme cleavage sites to clone into pGL3-basic firefly luciferase expression vector (Promega), forming STK11-pGL3 after enzymatic digestion.

Cell culture, transient transfections, and reporter gene assays

MCF-7 human breast cancer cells (ATCC) were maintained in 1:1 mixture of DMEM/F12 medium supplemented with 10% (v/v) FBS (Invitrogen), 1% penicillin and streptomycin. MCF-10A was maintained in DMEM/F12 media fortified with 1% HuMEC supplement (Gibco), 10% FBS and 1% penicillin and streptomycin (Invitrogen). 80% confluent MCF10 and MCF7 cells were subcultured in 24-well plates. Transfection of *STK11*-pGL3 constructs (800 ng) together with *Renilla* luciferase phRL-TK vector (200 ng) (Promega) was performed with Lipofectamine 2000 (Invitrogen). MCF7 and MCF10 cells were transfected with these vectors together with the *Renilla* luciferase phRL-TK vector to normalize for variation in transfection efficiency. After 24 h, Cells were lysed in 200 µl lysis buffer and the cell debris was removed by centrifugation. Reporter assays were performed using the dual luciferase assays system (Promega). Firefly luciferase activity was normalized by renilla luciferase activity, and the fold increase relative to pGL3-basic was calculated.

Statistical analysis

The level of expression in the MCF-7 cells was compared to the level of expression in MCF-10 cells. The relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). The data were presented as the mean $\pm$ SE. Statistical analyses were carried out using IBM SPSS version 22 statistical software (IBM SPSS, Chicago, IL, USA) and correlations between promoter and gene expression were carried out by Microsoft Excel®.

Independent samples were analysed using Student's *t* test using in silico online analysis software. Statistical differences were considered significant if the *p* value was less than 0.05 (denoted by stars **p* < 0.05; ***p* < 0.005; ****p* < 0.0005).

Results

Evaluation of *STK11* mRNA levels in MCF-7 and MCF-10 cells showed that *STK11* transcript was significantly more abundant in MCF-7 cells when compared to MCF-10 cells by reverse transcription PCR. The transcript was 6.63×10^{10} -fold abundant in MCF-7 cells relative to MCF-10 cells (*p* value ≤ 0.0005) (Fig. 1).

To determine whether changes in *STK11* mRNA levels could be transcriptionally regulated, a proximal promoter of the human *STK11* gene was analysed by transfecting the resultant *STK11*-PGL3 construct (Fig. 2a) into MCF-10 and MCF-7 cells. In MCF-10 cells, basal full-length *STK11* promoter activity was increased by 10.5-fold (*p* value = 0.003) compared to the promoter-less luciferase plasmid pGL3-Basic and 21.75-fold in MCF-7 cells (*p* value = 0.0013). Also, the promoter activity of *STK11* gene in MCF-7 cell was increased approximately twofold as compared to promoter activity of *STK11* in MCF-10 cell, the difference in reporter gene activity between the promoters in two cell lines was highly significant

Fig. 1 *STK11* gene expression in MCF-7 and MCF-10 cells. **a** The expression of *STK11* gene in the normal cell (MCF-10) was low compared to cancer cell (MCF-7) by reverse transcription. **b** *STK11* mRNA level was 6.63×10^{10} fold higher in MCF-7 than MCF-10 normal breast epithelial cells. ****p* value < 0.0005 student *t* test (the gene expression of *STK11* in MCF-7 relative to MCF-10)

Fig. 2 Luciferase activity in cells measured after 24 h of transient transfection. **a** Plasmid construct of *STK11*-pGL3, the dark black region is promoter fragment of 974 bp of *STK11* gene. **b** MCF-7 and MCF-10 cells were transfected with *STK11*-PGL3 and pRL-tk expression vectors. The values represent mean $\pm$ SD of three independent experiment. ***p* value < 0.005 student *t*-test (compare with promoterless activity), ****p* value < 0.0005 student *t* test (MCF-7 promoter activity compares with MCF-10 promoter activity). There was significant difference found between *STK11*-PGL3-MCF7 and *STK11*-PGL3-MCF10 cells (*p* value < 0.005)

(*p* value = 0.0005) (Fig. 2b). There was positive correlation between *STK11* expression and *STK11* promoter activity as demonstrated by Spearman non-parametric test (*p* = 0.048, $r^2 = 0.587$).

Discussion

Although prognosis of breast cancer patients is generally in their favor and many markers have been elucidated that improved adjuvant therapies still distant metastases is not uncommon. Also, women in advanced stage of the disease have a median survival rate of maximum 2 years only. *STK11* gene codes for both nuclear and cytoplasmic serine/threonine kinases. When nuclear signal of *STK11* was mutated to force it to remain in cytosol it retained its tumor suppressor activity in cytosol fully. Recent studies revealed that the expression of STKs is frequently altered in human cancers, suggesting that STKs may play an important role during cancer development (Fok et al. 2012). In light of

this, we investigated the functional role of *STK11* and have demonstrated that *STK11* plays a critical role in breast cancer. We found high expression of *STK11* gene in breast cancer cell line as compared to normal breast cell line by reverse as well as real-time PCR contradictory to the study conducted by Linher-Melville et al. 2012 who reported the less expression of LKB1 in MCF-7 cells due to ER α . Other studies reported that the multiple estrogen response elements (EREs) within the human *STK11* promoter region are important in the regulation of expression of *STK11* by ER α . MCF-7 breast cancer cell (ER- α positive) and have higher mRNA and protein level of *STK11* as compared to MDA-231 breast cancer cell (ER- α negative) (Brown et al. 2011). Our results are concomitant to the previous studies which reported that the *STK11* gene plays an important role in ER signaling in breast cancer cells and the activated expression of ER is associated to increased breast cancer risk (Nath-Sain and Marignani 2009). Furthermore, Upadhyay et al. reported that the kinase domain of *STK11* mutant can increase the stability of Polyomavirus enhancer activator 3 (PEA3) by interacting the E26 transformation-specific (ETS) domain of PEA3. Further, this interaction helped in the progress of carcinogenesis by the increased expression of the genes involved in metastasis in non-small cell lung cancers (NSCLC) (Upadhyay et al. 2006). Furthermore, under the stress, energy demand or energy supply imbalance conditions that are typical requirement of the tumor microenvironment leads to increase in the expression of AMPK that alters the cancer cell's flexibility to utilize nutrient glucose directly (Liang and Mills 2013; Herrmann et al. 2011). Other studies reported that the knockdown of *STK11* in MCF-7 and MDA-MB-435 cells induced migration and invasion of breast cancer cells due to which loss of cell polarity and cell-cell adhesion occurs (Li et al. 2014; Zhuang et al. 2006).

To investigate whether the change in expression is transcriptionally regulated, the promoter reporter assay was done to check the promoter activity of *STK11* gene. Our results showed that the promoter activity of *STK11* gene was significantly higher in MCF-7 as compared to MCF-10 cell. The increased promoter activity may be due to hypomethylation of CpG site in *STK11* promoter, which is in agreement with already reported studies on *STK31* gene. *STK31* was reactivated in gastrointestinal cancer by promoter hypomethylation, suggesting its potential role in cancer development (Yokoe et al. 2008). Further, the tumor suppressor genes P27, P53, and retinoblastoma (RB1) showed hypomethylation in promoter in odontogenic myxoma (OM), that lead to increase in the expression of these genes (Moreira et al. 2011). Other studies reported the increase in levels of total *STK11* mRNA and protein in MDA-MB-231 breast cancer cells cultured in the presence of hormone prolactin (PRL) (Linher-Melville et al. 2011)

leading to activation of *STK11* promoter by the binding of Signal transducer and activator of transcription (STAT) protein in gamma-activated sequence (GAS) site that was found in distal promoter of *STK11* (Linher-Melville and Singh 2014).

Conclusion

In conclusion, *STK11* is an important tumor suppressor gene that is activated in breast cancer. The mechanism of activation of gene is highly active promoter. This study can prove beneficial in treatment of breast and broad spectrum of cancers. *STK11* gene may serve as a potent and promising target for the development of control of expression directed therapy in breast cancer treatment.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interests regarding the publication of this paper.

References

- Baas AF, Smit L, Clevers H (2004) LKB1 tumor suppressor protein: PAraker in cell polarity. *Trends Cell Biol* 14:312–319
- Brown KA, McInnes KJ, Takagi K, Ono K, Hunger NI, Wang L, Sasano H, Simpson ER (2011) LKB1 expression is inhibited by estradiol-17 β in MCF-7 cells. *J Steroid Biochem Mol Biol* 127:439–443
- Esteller M, Avizienyte E, Corn P, Lothe R, Baylin S, Aaltonen L, Herman J (2000) Epigenetic inactivation of LKB1 in primary tumors associated with the Peutz–Jeghers syndrome. *Oncogene* 19:164–168
- Fok KL, Chung CM, Yi SQ, Jiang X, Sun X, Chen H, Chen YC, Kung HF, Tao Q, Diao R, Chan H, Zhang XH, Chung YW, Cai Z, Chang Chan H (2012) STK31 maintains the undifferentiated state of colon cancer cells. *Carcinogenesis* 33:2044–2053
- Furuyama T, Nakazawa T, Nakano I, Mori N (2000) Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. *Biochem Journey* 349:629–634
- Hearle N, Schumacher V, Menko FH, Olschwang S, Boardman LA, Gille JJ, Keller JJ, Westerman AM, Scott RJ, Lim W, Trimbatch JD, Giardiello FM, Gruber SB, Offerhaus GJ, de Rooij FW, Wilson JH, Hansmann A, Möslin G, Royer-Pokora B, Vogel T, Phillips RK, Spigelman AD, Houlston RS (2006) Frequency and spectrum of cancers in the Peutz–Jeghers syndrome. *Clin Cancer Res* 12:3209–3215
- Herrmann J, Byekova Y, Elmets C, Athar M (2011) The role of LKB1 in the pathogenesis of skin and other epithelial cancers. *NIH Public Access* 306:1–9
- Li J, Liu J, Yang J, Li P, Mao X, Li W, Liu P (2014) Loss of LKB1 disrupts breast epithelial cell polarity and promotes breast cancer metastasis and invasion. *J Exp Clin Cancer Res* 33:70

- Liang J, Mills GB (2013) AMPK: a contextual oncogene or tumor suppressor? *Cancer Res* 73:2929–2935
- Linhaer-Melville K, Singh G (2014) The transcriptional responsiveness of LKB1 to STAT-mediated signaling is differentially modulated by prolactin in human breast cancer cells. *BMC Cancer* 14:415
- Linhaer-Melville K, Zantinge S, Sanli T, Gerstein H, Tsakiridis T, Singh G (2011) Establishing a relationship between prolactin and altered fatty acid beta-oxidation via carnitine palmitoyl transferase 1 in breast cancer cells. *BMC Cancer* 11:56
- Linhaer-Melville K, Zantinge S, Singh G (2012) Liver kinase B1 (LKB1) expression is repressed by estrogen receptor (ER α) in MCF-7 human breast cancer cells. *Biochem Biophys Res Commun* 417:1063–1068
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real time quantitative PCR and the 2 ($- \Delta \Delta$ CT) method. *Methods* 25:402–408
- Mantovani R (1999) The molecular biology of the CCAAT-binding factor NFY. *Gene* 239:15–27
- Moreira P, Cardoso F, Brito J, Batista A, Gomes C, Gomez R (2011) Hypomethylation of tumor suppressor genes in odontogenic myxoma. *Braz Dent J* 22:422–427
- Nath-Sain S, Marignani PA (2009) LKB1 catalytic activity contributes to estrogen receptor alpha signaling. *Mol Biol Cell* 20:2785–2795
- Peutz J (1921) On a very remarkable case of familial polyposis of mucous membrane of intestinal tract and nasopharynx accompanied by peculiar pigmentation of skin and mucous membrane. *Nederl Maandschr Geneesk* 10:134–136
- Suske G (1999) The Sp-family of transcription factors. *Gene* 238:291–300
- Upadhyay S, Liu C, Chatterjee A, Hoque MO, Kim MS, Engles J, Westra W, Trink B, Ratovitski E, Sidransky D (2006) LKB1/STK11 suppresses cyclooxygenase-2 induction and cellular invasion through PEA3 in lung cancer. *Cancer Res* 66:7870–7879
- Yokoe T, Tanaka F, Mimori K, Inoue H, Ohmachi T, Kusunoki M, Mori M (2008) Efficient identification of a novel cancer/testis antigen for immunotherapy using three-step microarray analysis. *Cancer Res* 68:1074–1082
- Zhong D, Liu X, Khuri FR, Sun SY, Vertino PM, Zhou W (2008) LKB1 is necessary for Akt-mediated phosphorylation of proapoptotic proteins. *Cancer Res* 68:7270–7277
- Zhuang Z-G, Di G-H, Shen Z-Z, Ding J, Shao Z-M (2006) Enhanced expression of LKB1 in breast cancer cells attenuates angiogenesis, invasion, and metastatic potential. *Mol Cancer Res* 4(11):843–849