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Original Article

Expression of p-AMPK is associated with hormone receptor phenotypes and lymph node metastasis in breast cancer

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Abstract: Many studies have investigated the role of phosphorylated AMP-activated protein kinase (p-AMPK) in cancer tumorigenesis and its antineoplastic effects in cancer models. The aim of this study was to examine the association of p-AMPK immunohistochemical expression with the clinicopathological parameters in breast cancer. Methods: 449 cases of previously diagnosed breast cancer, and 27 tissue samples of fibroadenomas and normal breast were utilized for detection of p-AMPK expression using tissue microarrays and immunohistochemistry. Results: Nuclear and cytoplasmic immunoexpression of p-AMPK was identified in 374 (83.3%) of breast cancer and 25 (92.6%) control cases. Hormone receptor phenotypes are significantly associated with p-AMPK staining only in epithelial cells (*P*-value = 0.033); the proportions of ER+ PR- HER2- hormone receptor phenotype were significantly higher in positive p-AMPK staining epithelial cells. Loss of progesterone receptor (PR) expression was noticeable in breast tumors with positive p-AMPK stromal cells (*P*-value = 0.043). Histotype of breast cancer is significantly associated with p-AMPK staining in stromal cells only; positive p-AMPK staining was more prevalent in DCIS and ductal histotypes (*P*-value = 0.032). Lymph node involvement was also significantly associated with p-AMPK immunostaining in both epithelial cells and stromal cells (*P*-value = 0.037 and *P*-Value = 0.042 respectively). No significant differences in survival behavior were observed and no significant associations were detected with tumor size, grade of disease, stage, vascular invasion, margins involvement and disease recurrence. Conclusions: Our results showed a slight decrease in p-AMPK expression in breast cancer in comparison with control group. Expression of p-AMPK could be a useful marker in the diagnosis and prognosis of some types of breast cancer with certain hormone receptor phenotype and lymph node involvement.

Keywords: Phosphorylated AMP-activated protein kinase, p-AMPK, breast cancer, immunohistochemistry

Introduction

Adenosine monophosphate activated protein kinase (AMPK) is a complex molecule made out of three subunits, of which α is a catalytic subunit, β and γ are regulatory subunits. There are twelve possible isoforms of AMPK in humans because each subunit is encoded by 2 or 3 different genes (α1, α2, β1, β2, γ1, γ2, γ3). This protein kinase is an energy sensor inside cells. Activation of AMPK occurs through the addition of phosphate group at threonine (Thr-172); this process is catalyzed by different kinase enzymes such as LKB1 and CaMKK as a consequence to the stresses of metabolism, which increase ATP consumption (muscle contraction) or inhibit ATP production (hypoxia, ischemia, and glucose deficiency) and accordingly raise

Table 1. Describe the distribution of various clinicopathological variables with p-AMPK immunostaining in breast cancer

		p-AMPK immunostaining in Epithelial cells				*P*-Value	p-AMPK immunostaining in Stromal cells				*P*-Value
		Negative		Positive			Negative		Positive		
		Count	%	Count	%		Count	%	Count	%	
Group	p-AMPK in breast cancer	75	16.7%	374	83.3%	0.203	82	18.3%	367	81.7%	0.151
	p-AMPK in Control group	2	7.4%	25	92.6%		2	7.4%	25	92.6%	
Age in Years	<40	14	20.3%	55	79.7%	0.089	14	20.3%	55	79.7%	0.021
	40-49	27	23.7%	87	76.3%		31	27.2%	83	72.8%	
	50-59	16	11.9%	118	88.1%		15	11.2%	119	88.8%	
	60-69	8	11.8%	60	88.2%		10	14.7%	58	85.3%	
	≥70	10	19.6%	41	80.4%		11	21.6%	40	78.4%	
	NA	0	0.0%	13	100.0%		1	7.7%	12	92.3%	
Hormone receptor phenotype	ER- PR- HER2-	10	14.5%	59	85.5%	0.033	9	13.0%	60	87.0%	0.221
	ER- PR- HER2+	14	20.6%	54	79.4%		13	19.1%	55	80.9%	
	ER- PR+ HER2-	4	57.1%	3	42.9%		3	42.9%	4	57.1%	
	ER- PR+ HER2+	2	22.2%	7	77.8%		2	22.2%	7	77.8%	
	ER+ PR- HER2-	3	6.0%	47	94.0%		4	8.0%	46	92.0%	
	ER+ PR- HER2+	3	17.6%	14	82.4%		3	17.6%	14	82.4%	
	ER+ PR+ HER2-	21	14.6%	123	85.4%		28	19.4%	116	80.6%	
	ER+ PR+ HER2+	18	21.2%	67	78.8%		20	23.5%	65	76.5%	
ER	ER-	30	19.6%	123	80.4%	0.236	27	17.6%	126	82.4%	0.808
	ER+	45	15.2%	251	84.8%		55	18.6%	241	81.4%	
PR	PR-	30	14.7%	174	85.3%	0.300	29	14.2%	175	85.8%	0.043
	PR+	45	18.4%	200	81.6%		53	21.6%	192	78.4%	
HER	HER2-	38	14.1%	232	85.9%	0.067	44	16.3%	226	83.7%	0.185
	HER2+	37	20.7%	142	79.3%		38	21.2%	141	78.8%	
Lymph node involvement	Negative	39	21.1%	146	78.9%	0.037	42	22.7%	143	77.3%	0.042
	Positive	36	13.6%	228	86.4%		40	15.2%	224	84.8%	
Size of tumor	< 2	10	16.7%	50	83.3%	0.999	10	16.7%	50	83.3%	0.642
	2-5	47	16.7%	234	83.3%		55	19.6%	226	80.4%	
	> 5	18	16.7%	90	83.3%		17	15.7%	91	84.3%	
Grade	I	13	17.8%	60	82.2%	0.757	15	20.5%	58	79.5%	0.779
	II	37	15.5%	202	84.5%		41	17.2%	198	82.8%	
	III	25	18.2%	112	81.8%		26	19.0%	111	81.0%	
Histotype	DCIS	1	5.9%	16	94.1%	0.164	3	17.6%	14	82.4%	0.032
	DUCTAL	67	16.3%	343	83.7%		71	17.3%	339	82.7%	
	DUCTAL+MUCINOUS	3	33.3%	6	66.7%		5	55.6%	4	44.4%	
	LOBULAR	4	30.8%	9	69.2%		3	23.1%	10	76.9%	
Stage	I	10	20.0%	40	80.0%	0.184	10	20.0%	40	80.0%	0.080
	II (a)	29	22.1%	102	77.9%		32	24.4%	99	75.6%	
	II (b)	21	15.1%	118	84.9%		26	18.7%	113	81.3%	
	III	9	13.6%	57	86.4%		8	12.1%	58	87.9%	
	IV	6	9.5%	57	90.5%		6	9.5%	57	90.5%	
Vascular Invasion	Negative	55	17.8%	254	82.2%	0.355	63	20.4%	246	79.6%	0.083
	Positive	20	14.3%	120	85.7%		19	13.6%	121	86.4%	
Margins	Negative	64	16.3%	329	83.7%	0.529	72	18.3%	321	81.7%	0.993
	Positive	11	19.6%	45	80.4%		10	17.9%	46	82.1%	
Recurrence	No	66	16.5%	333	83.5%	0.794	73	18.3%	326	81.7%	0.959
	Yes	9	18.0%	41	82.0%		9	18.0%	41	82.0%	

the AMP/ATP ratio [1-3]. It has been confirmed that AMPK restrains basically all anabolic metabolic processes that support cellular proliferation, for instance rRNA and protein synthesis as well as fatty acid and phospholipid synthesis [1, 4]. Accordingly, it is not unexpected that AMPK antagonizes tumor development and progression. AMPK influences cell proliferation and arbitrates cell cycle checkpoints of neoplastic cells following depletion of energy sources. When stimulated, phosphorylated AMPK (p-AMPK) suppresses energy-depleting pathways such as cell proliferation and promotes energy- generating catabolic processes such as fatty acid oxidation, uptake of glucose and glycolysis [1, 4-6]. Additional anti-neoplastic influences of AMPK could involve increasing autophagy and DNA repair after ultraviolet ray injury [7]. This opinion was strengthened and confirmed by a set of studies, which has found that AMPK motivates and phosphorylate p53 [8] and p21 [9], thus distracting the cell cycle and promoting cellular survival. AMP-activated protein kinase has been found to suppress mTOR (mammalian target of rapamycin) and thereby limits protein biosynthesis. Furthermore, activated AMPK, in cancer cells suppresses lipogenic enzymes such as fatty acid synthase (FA) and acetyl CoA carboxylase (ACC), which are greatly found in neoplastic compartment due to the increased request for fatty acids which are needed to be integrated in the dividing cells' cytoplasmic membranes [1, 4, 10].

There are several studies and reviews which have summarized the role of AMPK activation in cancer development and progression [1, 4, 11-16], and some studies have associated AMPK activation with better prognosis among different types of cancer including gastric [17], colorectal [18], head and neck [19], kidney [20], lung [21], and liver [22]. However, to the best of our knowledge, there have been four studies, which investigated the immunohistochemical expression of p-AMPK in breast cancer, of which two studies only reported the relation between p-AMPK expression and clinicopathological parameters of breast cancer, but with conflicting results [23-25]. Therefore, the present research study defines the p-AMPK immunohistochemical expression in breast tumors, and assesses the association between p-AMPK expression patterns and the clinicopathological findings and follow-up data of female population of the west province in Saudi Arabia.

Materials and methods

Four hundred and forty nine cases of mammary tumors were recovered from the stores of the Pathology Department at King Abdulaziz University Hospital, Kingdom of Saudi Arabia. In addition, 27 cases of normal breast tissues and fibroadenomas were used as controls. Paraffin tissue blocks were sectioned and H&E stained for tumor histological evaluations. The clinical data of patients such as age, histotype, size, stage and grade were retrieved from the unit of medical records (**Table 1**). World Health Organization (WHO) recommendations regarding grade and stage of breast tumors were applied. All breast cancer, fibroadenomas and normal tissue blocks were used for tissue microarray production. The current research study has met all the instructions and the requirements of the obtained ethical committee approval.

Tissue microarray production

Four hundred and forty nine cases of breast carcinoma and twenty seven cases of normal breast tissue and fibroadenoma were utilized to construct tissue microarray (TMA) as we reported in our previous study [26]. TMA blocks were sectioned and put on coated slides and then subjected to p-AMPK immunohistochemical staining.

Immunohistochemical staining method

Multimeric technology was used in the immunohistochemistry staining of breast tumor sections employing p-AMPKα1/2 (Thr 183/172) rabbit polyclonal antibody (1:100 dilution; product code: sc-101630, Santa Cruz Biotechnology, INC, Dallas, USA), and ULTRAVIEW TM DAB visualizing system. BenchMark ULTRA autostainer was employed for automated immunohistochemistry staining (Ventana, Arizona, USA). A negative control slide has been added to each staining run, this slide contained Tris buffer instead of the primary antibody. Hep G2 cell lysate (product code: sc-2227, Santa Cruz Biotechnology, INC, Dallas, USA) was involved as positive control. Cases with positive staining in more than 5% of neoplastic cells were counted positive.

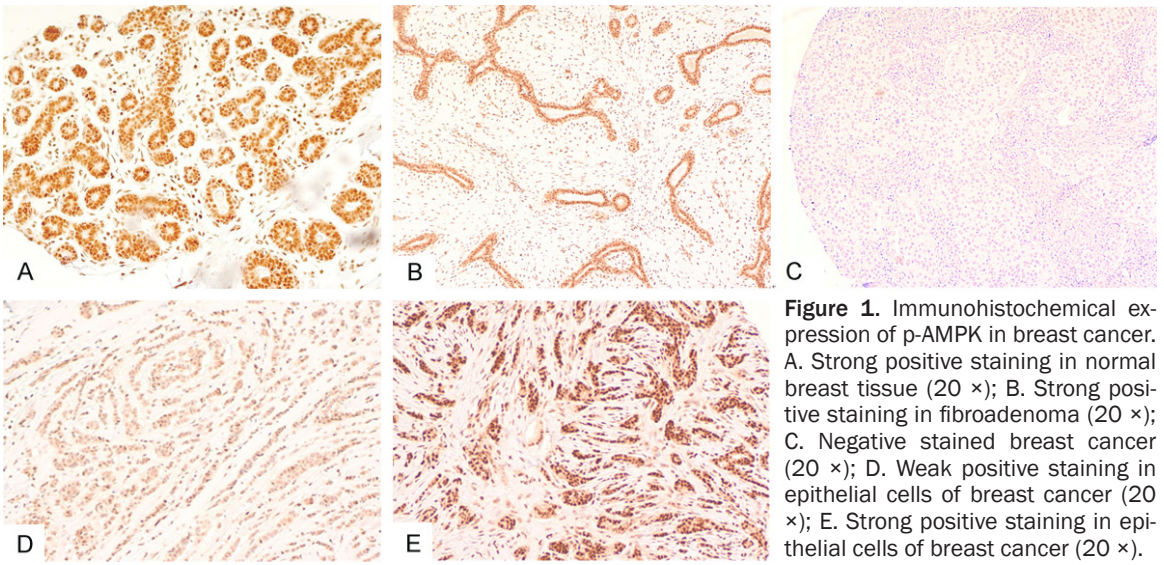

Figure 1. Immunohistochemical expression of p-AMPK in breast cancer. A. Strong positive staining in normal breast tissue (20 ×); B. Strong positive staining in fibroadenoma (20 ×); C. Negative stained breast cancer (20 ×); D. Weak positive staining in epithelial cells of breast cancer (20 ×); E. Strong positive staining in epithelial cells of breast cancer (20 ×).

P-AMPK immunoreactivity was scored, for staining intensity and percentage of positively stained cells by two pathologists. The frequency of positively stained cells has been evaluated using semi-quantitative approach in three 400 magnification fields. Positive p-AMPK breast cancer cases have been scored for staining intensity considering strong = 3, moderate = 2, weak staining = 1, and negative = 0. Immunostaining scores have been introduced as negative (absence of staining: score = 0) and positive (scores 1, 2 and 3) in the current study. When a difference between the scores of two pathologists has occurred, the smallest score of staining was recorded.

Statistical analysis

Results were evaluated using version 21 IBM-SPSS software. Categorical data has been shown as incidences and percentages. Chi-Square test is applied to explore the association of p-AMPK immunostaining with various clinicopathological variables of breast cancer. Breslow (Generalized Wilcoxon) test is applied to compare survival distributions for the different levels of p-AMPK immunostaining. *P*-value < 0.05 is counted as significant.

Results

Four hundred and forty nine cases of breast carcinoma were reviewed. Clinicopathological findings of these neoplasms are reported in **Table 1**. Invasive ductal carcinoma (91.3%) was the most common type and less frequently, ductal carcinoma in situ (3.8%), invasive lobular carcinoma (2.9) and mucinous carcinoma (2%) (**Table 1**). Patients' age ranged from 24 to 94 years with median of 50.7 years.

Nuclear and cytoplasmic p-AMPK immunoexpression was detected in the epithelial cells of 374 (83.3%) and in stromal cells of 367 (81.7%) breast cancer cases respectively. Twenty five (92.6) control cases showed p-AMPK immunoexpression in both epithelial and stromal cells (**Figure 1**). No statistical significant difference was noted between breast carcinoma cases and control group regarding p-AMPK immunoexpression. **Table 1** describes the distribution of p-AMPK immunostaining detected in transformed epithelial cells and stromal cells of breast carcinoma with various clinicopathological variables. More than 80% of breast tumors showed positive p-AMPK staining in more than 50% of tumor cells. About 85% of positive p-AMPK breast cancer cases showed weak cytoplasmic staining and moderate to strong nuclear immunoreactivity in both epithelial and stromal cells.

Hormone receptor phenotypes are significantly associated with p-AMPK immunostaining in epithelial cells only (*P*-value = 0.033). The proportion of breast tumors with ER+ PR- HER2- phenotype is significantly higher in breast tumors with positive p-AMPK epithelium. Loss of progesterone receptor (PR) expression was noticeable in breast tumors with positive p-AMPK stromal cells (*P*-value = 0.043). Histotype of breast cancer was significantly associated

with p-AMPK immunostaining in stromal cells only; DCIS and ductal histotypes are more prevalent in positive p-AMPK tumor cases (*P*-value = 0.032). Lymph node involvement was also significantly associated with both p-AMPK immunostaining in epithelial and stromal cells (*P*-value = 0.037 and *P*-Value = 0.042 respectively). No significant associations were detected with tumor size, grade of disease, stage, vascular invasion, margins and disease recurrence.

Breslow (Generalized Wilcoxon) test has been applied to compare the survival distribution of negative and positive cases of p-AMPK immunostaining in epithelial cells and stromal cells. No significant differences in survival behavior were observed; however, weak significant difference in survival adjusted for recurrence is observed in positive epithelial cells (0.079). Relatively poor survival is observed with positive epithelial cells.

Discussion

Breast tumors are the most common malignancy among the female population with around 1,700,000 new cases and over 580,000 deaths of breast tumors in the USA in 2014 [American Cancer Society, 2014. Cancer Facts & Figures 2014. Cancer Facts Fig. 1-72.http://www.cancer.org/research/cancer-facts-statistics/all-cancer-facts-figures/cancer-facts-figures-2014.html]. Breast cancer has a comparable rank among female neoplasms accounting for (25.8%) of all diagnosed tumors in the female population in Saudi Arabia in 2012 [Cancer Incidence Report Saudi Arabia 2012, Saudi Health Council, Saudi Cancer Registry. http://www.chs.gov.sa/En/HealthRecords/CancerRegistry/Pages/CancerRegistryRecords.aspx]. Regardless of significant innovations in the diagnosis and management of breast tumors, the disease still represents a great challenge to health professionals because of bad prognosis and elevated relapse rate in some breast cancer histotypes especially triple negative, as 30-40% of new cancer cases recur within 5 years [27]. The treatment of breast cancer depends immensely on the patients' clinicopathological findings, such as tumor grade, and TNM stage as meters of prognosis. However, these parameters are not adequate to expect patients' clinical outcome and worse yet, may cause inconsistencies in a group of tumor with similar stage or grade. This is mainly related to heterogeneity of breast cancer cells [28]. Hence, there is a necessity to obtain new diagnostic biomarkers and therapeutic modalities in order to assist in disease diagnosis, define prognosis, improve high risk patients' stratification and enhance clinical outcomes [29].

In the last decade, increased awareness has been brought to the role of p-AMPK as a hopeful marker in tumor prevention, and likely therapeutic target in numerous human neoplasms, making p-AMPK an important topic in tumor biology. Many papers have described p-AMPK immunoexpression profile and reported its significant association with clinicopathological factors in many human malignancies including gastric cancer [17], colorectal carcinoma [18], hepatocellular carcinoma [22], lung cancer [21], squamous cell carcinoma of the head and neck [19], and renal cell carcinoma [20]. There have been also four studies which assessed p-AMPK expression in tumors and benign conditions of breast [23-25, 30], of which only two studies described the relation between p-AMPK immunophenotype and clinicopathological factors of breast cancer [25, 30]. The results of these studies showed inconsistent relationships with clinicopathological parameters. The other two studies of Duchnowska and colleagues [23] and Hadad and colleagues [24] reported the mechanisms of metformin action via up-regulation of tumor p-AMPK, and downstream signaling pathways-AMPK/mTOR and Ras/Raf/MAPK in vivo in breast cancer patients.

The findings of the present study reach the same conclusion of Hadad et al. [30] who found decreased expression in breast cancer compared to breast normal tissue. Regarding breast cancer, the current study is the first to report an association of p-AMPK expression with hormone receptor phenotype status (ER+ PR- HER2-) in breast cancer. The present investigation found significant association between increased immunoexpression of p-AMPK and lymph node metastasis, a finding which is opposite to the results of Hadad and colleagues [30] who reported inverse relationship and also contradicts the findings of Zhang and coworkers who could not find a relationship [25]. Although Hadad et al. [30] reported that increased p-AMPK immunoexpression was correlated

with low tumor grade and/or stage, our study and similarly that of Zhang and coworkers' study [25] could not find a statistical significant association between p-AMPK expression and tumor grade or stage of breast cancer. However, in the present study, the remarkable increase in p-AMPK immunoexpression in advance stages could be of clinical significance, which might support the mechanistic role of p-AMPK in breast cancer progression, and cancer cell survival. Furthermore, it proposes that, in particular conditions, the generally accepted role of activated AMPK as a tumor suppressor could be subverted by neoplastic cells by appropriating p-AMPK to gain a cellular growth opportunity [31-36].

Alternatively, AMPK might operate as a double molecule in tumor growth and progression based on several factors such as AMPK isoform and its level of activation in addition to other activated compensatory cellular processes. It is feasible that moderate AMPK activation by moderate stress could utilize protecting powers and produce an oncogenic-like behavior, while extreme stress could possibly stimulate AMPK showing suppressing activities and causing tumor cell death.

Therefore, further studies investigating the role of activated AMPK in breast cancer will elucidate the mechanisms that lie behind the role of AMPK in protecting cancer cells and avoiding stress injury.

Thus, expression of p-AMPK could be a useful marker in the diagnosis and prognosis of some types of breast cancer with certain hormone receptor phenotype and lymph node involvement. The correlation of p-AMPK with some clinicopathological parameters may suggests the involvement of this molecule in breast tumor progression and cell survival.

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Disclosure of conflict of interest

None.

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