EXPRESSION ANALYSIS AND RELATIONSHIP OF PROTEIN KINASE CK2α WITH HISTOPATHOLOGICAL FEATURES IN HUMAN BREAST CANCER

Fatima Qaiser[1✉], Abdul Khaliq Naveed[2], Sarah Sadiq[3], Palvasha Waheed[3], Iqbal Muhammad[4], Rubina Younis[4], Shoaib Naiyar Hashmi[5]

ABSTRACT

OBJECTIVES: The present study was designed to evaluate the role of CK2α; in prognosis of breast cancer patients diagnosed at the same stage of the disease, and to predict aggressiveness of the tumor.

METHODS: A retroprospective immunohistochemical analysis of CK2α was carried out in human breast cancer tissue specimens. All the cases included were diagnosed at stage II of disease. χ^2 tests and Regression analysis were carried out to determine the correlation between histopathological parameters and expression pattern of CK2α

RESULTS: There was a positive correlation between total as well as nuclear expression of CK2α with increasing Nottingham prognostic index ($p<0.0001$). CK2α was also found to be significantly associated with the lymph node metastasis ($p<0.0001$).

CONCLUSION: Immunohistochemical expression of CK2α can be used as an indicator of poor prognosis of disease even if overall stage of the breast cancer is the same. CK2α can also serve to predict the aggressiveness of the breast cancer as well and can identify the sub set of patients at high risk of developing lymph node metastasis.

KEY WORDS: CK2alpha protein (MeSH), Nottingham prognostic index (Non-MeSH), Immunohistochemistry (MeSH), Prognosis (MeSH), Breast cancer (MeSH), Neoplasm Metastasis (MeSH)

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[1✉] Department of Biochemistry and Molecular Biology, Army Medical college, National University of Sciences and Technology, 44000, Islamabad, Pakistan
E-mail: fatimaqaisar@amcollege.nust.edu.pk; fatima_qsr@yahoo.com
[2] Islamic International Medical College Riphah International University Al-Mizan Campus, 274 Peshawar Road Rawalpindi – Pakistan
[3] Department of Biochemistry and Molecular Biology, Army Medical college, National University of Sciences and Technology, Islamabad, Pakistan
[4] Department of Histopathology, Army Medical college, Rawalpindi, Pakistan
[5] Department of Histopathology, Armed Forces Institute of Pathology, Combined Military Hospital, Rawalpindi, Pakistan
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INTRODUCTION

Breast cancer is one of the leading causes of death in women. According to "World Cancer Report" International Agency for Research on Cancer, breast cancer accounts for 25% of all cancers in women, excluding skin cancer (non-melanoma). There is a growing consensus around the world that underlying molecular and biological processes are critical for diagnosis, prognosis, better management and treatment of breast cancer patients.[1] In this regard efforts are made to discover a clinically significant biomarker. Research has been done to elucidate the signaling pathways involved in the initiation and progression of breast cancer.

Protein Kinase CK2 a serine/threonine kinase is a quaternary heterotetrameric holoenzyme, with two catalytic (αα') and two regulatory (β) subunits.[2] The relationship of CK2 with cancer has been long recognized and CK2 has been found to be uniformly over expressed in all the cancers studied so far.[3-5] Role of CK2 in pathogenesis of breast cancer has been documented by many research groups. Mouse model of breast cancer expressing CK2 shows several features similar with human breast cancer. Only the slight over expression of CK2α transgene evokes an ongonenic potential in breast cancer mice model.[6] Furthermore, increased CK2 activity and proteins levels were observed in chemically induced mammary tumors, suggesting a pathological role of CK2 in breast cancer neoplasia. Enhanced CK2 activity has been observed in both; samples from primary breast cancer patients as well as breast cancer cell lines.[7] Inhibition of CK2 in breast cancer and other cell lines have shown induction of apoptosis and cell death.[8-9]

So far, to our knowledge there is just one major immunohistochemical study describing the potential prognostic role of CK2α in human breast cancer specimens.[1] There are studies on prostate[10,11] and head and neck cancer[12,13] which strengthen the notion in favor of CK2α as a prognostic marker for cancer.[14]

Over the past decade, emphasis on importance of personalized medication for treating breast cancer has grown tremendously. Gene profiling is considered as the most appropriate test for customized treatment but it has a limited use in clinical settings. One

recent study has demonstrated that even the annual mammography is not of much help for the in time diagnosis. In fact, this study showed that there was no clear advantage of regular mammography over the physical examination. Regular mammography checks did not decrease the mortality rate from breast cancer, instead 22% of the cases were over diagnosed and thus over treated.[15] Subsequently, there is an increase in an interest and demand for the search of immunohistochemical markers. These markers are more relevant and better adaptable in a clinical practice, they can help in clear classification of breast cancer, can help in better prognosis of the disease and can distinguish biologically different subtypes of breast cancer that behave distinctively. This will greatly improve the management and treatment options for breast cancer patients. Thus the present study is designed to evaluate the role of CK2α in prognosis of breast cancer. We want to determine the association of CK2α with the pathological features of the breast cancer patient that were already classified at stage II of the disease. The main aim of the present study is to further classify these patients into a subset that might behave distinctively and to predict the aggressiveness of tumor and identification of the high risk patients. In this regard, we will test the hypothesis that CK2α is associated with the increasing NPI and lymph node metastasis in breast cancer. Patients that are at the same stage of breast cancer will be evaluated to see whether the over-expression of CK2α is associated with the increasing Nottingham prognostic index, poor histopathological findings and other associated complications such as metastasis.

METHODS

Sample Collection

A total of 100 paraffin embedded tissue samples were obtained from the tissue repository of Armed Forces Institute of pathology (AFIP). All the cases included were already diagnosed at stage II of breast cancer. All the samples included were collected within the same year and none of the samples were older than a year

Antibody and Chemicals

CK2α (C-18 goat polyclonal, Santa Cruz Biotechnology cat# 6479, 1:200) was used to carry out immunohistochemistry. LSABKIT /HRP Rb/Mo/Goat (DAB+) from Dako, peroxidase-based visualization kit was used as a detection system. All the other procedural chemicals for IHC were purchased from DAKO or mentioned otherwise

Immunohistochemistry

Immunohistochemistry was carried out manually at AFIP. Sections were cut at 3-4 micron. Each section was transferred to positively charged slides with a histogrip coating. Slides were placed in oven for 1 hr at 56°C for fixation. De-waxing was carried out in absolute xylene for 7-10 min. Slides were rehydrated in ascending grades of alcohol (absolute, 80% and 70% alcohol for 2-3 min each). Finally slides were dipped in water to complete rehydration. Antigen retrieval was carried out in 10X EDTA + TRIS (antigen retrieval solution; 10mM Tris Base, 1mM EDTA Solution, 0.05% Tween 20, pH 9.0) at 100°C for 25 min in an electric decloaking chamber. Hydrogen peroxidase (in phosphate buffer saline) solution (blocking solution) was added for 5 min. Washing was carried out with PBS (3 times, 5 min each time). Slides were incubated with primary antibody (diluted in PBS) for 1 hr followed by treatment with secondary antibody for 15 min. Streptavidin-HRP was then added for 15 min followed by addition of chromogen DAB for 10 min. Slides were washed with distilled water and counter stained with hematoxylin for 1 min. Once staining was completed slides were dehydrated in descending grades of alcohol and xylene (90%, 80% and 70%) and were mounted with cover slips coated with Distrene, Plasticiser, Xylene DPX.

Scoring

Each slide was scored blindly by three histopathologists. In case of discrepancy, the two nearest readings were considered. For CK2α, the range of score is from 0-3+ for nuclear and cytoplasmic stain, where 0 is no staining, +1 is weak staining, +2 is moderate staining and 3+ is the maximum staining (11) . Sum of these two readings is the total score for CK2α.

Statistical Analysis: Chi square test was used to determine association between pathological features of breast cancer patients, Nottingham prognostic index, lymph node metastasis and expression pattern of CK2α. Regression analysis was carried out to find the relationship between the CK2α and size of tumor. Confidence interval was set at 95% and P value less than 0.05 was considered significant, unless otherwise stated.

Ethical Approval: The study does not involve the direct patient/human involvement. No personal data of any patient is being shown in the study. The present study is reviewed and approved by Ethical Review Committee, Armed Forces Institute of Pathology (AFIP). The study followed the Good Clinical Practices as approved by FDA, 1996 and Declaration of Helsinki (WMA, 2000)

RESULTS

Cliniocopathlogical Features of Breast Cancer Patients

The observations obtained from the data base of patients at department of histopathology at AFIP are shown in Table I. All the clinical and pathological grades were assigned by the classified

TABLE 1: CLINICOPATHOLOGICAL FEATURES OF BREAST CANCER PATIENTS

Clinicopathological Features	Measurements
Mean Age	50.0±13(29-80) years
Nottingham prognostic index	5.4 (3-8)
3-5	50%
6-9	50%
Lymph node Metastasis	
Present	65%
Absent	35%
Skin Involvement	
Present	53%
Absent	47%
Lympho Vascular involvement	
Present	65%
Absent	35%
Size of Tumor cm3	8.9 ±5 cm3 (3-25.5)

Values are represented as mean for age, size of tumor and Nottingham prognostic index. Actual values are given for skin involvement/lymphovascular invasion and Lymphnode Metastasis.

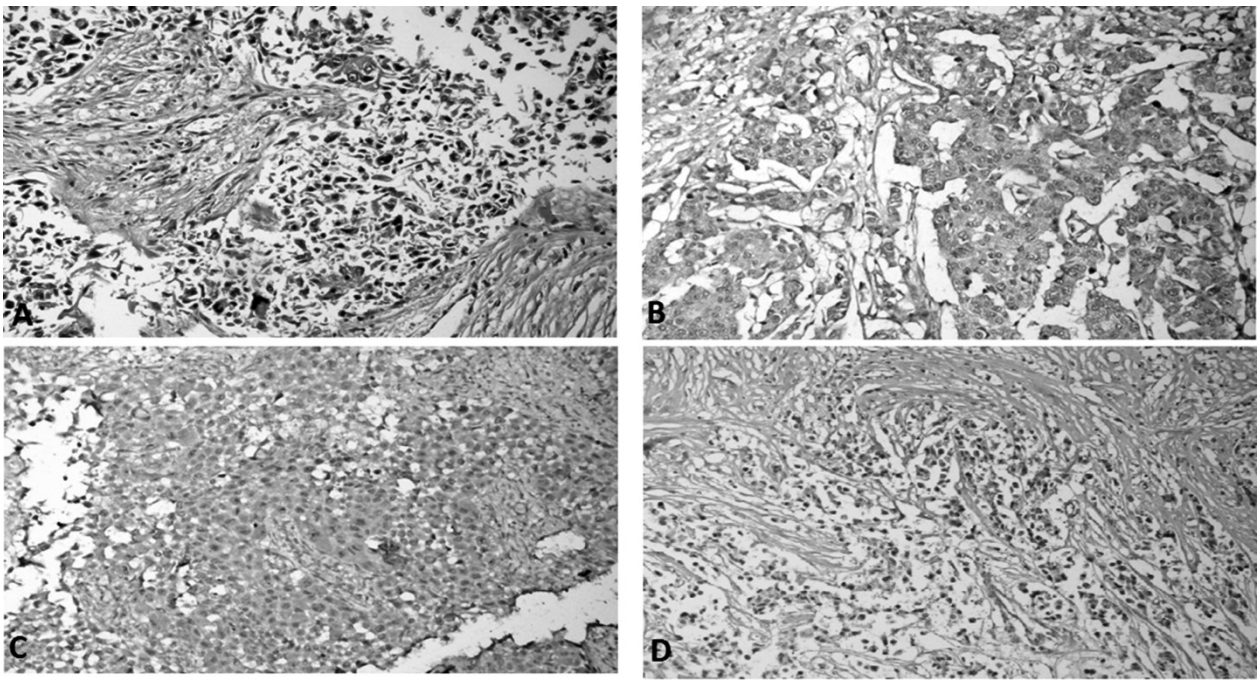

Figure I: Immunohistochemical Expression pattern of CK2α

Breast cancer tissue samples were stained using the DAB method to determine the expression pattern of CK2α. Acquisition of brown color shows the positive staining. CK2 was scored in both nuclear and cytoplasmic compartment. Figure A shows the representative H & E staining. Figure B shows nuclear 1+ staining with 2+ cytoplasmic, this makes total score of CK2α as +3. Figure C shows the positive staining with scores as nuclei 2+ and cytoplasmic 2+ & total CK2α score of 4+. Figure D scoring is nuclei 3+, cytoplasmic 1+ & total CK2α score of 4+

TABLE II: ASSOCIATION OF CK2α IMMUNE-STAINING WITH NOTTINGHAM PROGNOSTIC INDEX

	Nuclear CK2α staining scores		
Nottingham prognostic index			
	0/+1	+2/+3	
3-5	33(22.5)	17 (27.5)	P<0.0001*
6-9	12(22.5)	38 (27.5)	
Cytoplasmic CK2α staining scores			
	0/+1	+2/+3	
3-5	33 (31.5)	17 (18.5)	P=0.53
6-9	30 (31.5)	20 (18.5)	
Total CK2α staining scores			
	+1/+2	+3 and above	
3-5	27 (19)	23 (31)	P<0.0001*
6-9	11 (19)	39 (31)	

Association of CK2α with Nottingham prognostic index was carried by chai-square test. Numbers outside the parenthesis are the actual numbers and numbers with in the parenthesis represent the calculated expected value. Confidence interval was set at 95% and P<0.05 was considered significant. *represents significant association.

TABLE III: RELATIONSHIP BETWEEN CK2α IMMUNOSTAINING AND BREAST CANCER METASTASIS:

	Nuclear CK2α staining scores		
Lymph node Metastasis			
	0/+1	+2/+3	
Present	16 (29.25)	49 (35.75)	P<0.0001*
Absent	29 (15.75)	6 (19.25)	
Cytoplasmic CK2α staining scores			
	0/+1	+2/+3	
Present	38 (40.95)	27 (42.05)	P=0.2
Absent	25 (22.05)	10 (12.95)	
Total CK2α staining scores			
	+1/+2	+3 and above	
Present	16 (24.7)	49 (40.3)	P=0.0001*
Absent	22 (13.3)	13 (21.7)	

Relationship between these two parameters was determined by chai-square test. Numbers outside the parenthesis are the actual numbers and numbers with in the parenthesis represent the calculated expected value. Confidence interval was set at 95% and P<0.05 was considered significant. * represents significant association

histopathologist. Hematoxylin and eosin (H&E) staining was carried out on every sample to determine the pathological stage of the tumor.

Expression Analysis of CK2α in Breast cancer tissue specimens.

To determine the expression pattern (cytoplasmic and nuclear) of CK2α, immunohistochemistry was carried out. Staining was scored. For CK2α range of score is from 0-3+ for nuclear and cytoplasmic stain. Sum of these readings is the total score for CK2α, Figure I (A, B, C and D). CK2α was over expressed in all the cancer tissues examined.

Assessing role of CK2 in prognosis of disease; Correlation of CK2α with clinicopathological features

Immunohistochemical analysis was carried to determine the relationship between expression pattern of CK2α and clinicopathological features (Nottingham prognostic index, lymphovascular inva-

TABLE IV: RELATIONSHIP BETWEEN CK2α IMMUNOSTAINING AND CLINICAL AND PATHOLOGICAL CHARACTERS OF HUMAN BREAST CANCER:

	Nuclear CK2α	Cytoplasmic CK2α	Total CK2α
Size of Tumor cm3	R= 0.14, p=0.14	R=0.12, p=0.2	R=0.01, p=8
	Nuclear CK2α	**Cytoplasmic CK2α**	**Total CK2α**
Lymphovascular Invasion	χ2 p=0.002*	χ2 p=0.2	χ2 p=0.6
	Nuclear CK2α	**Cytoplasmic CK2α**	**Total CK2α**
Skin Involvement	χ2 p=0.05	χ2 p=0.8	χ2 p=0.4

Regression analysis was carried out to determine the relationship between size of the tumor and CK2α scores. For all the other pathological features chai-square was done. P<0.05 was considered significant for all the parameters. *shows statistically significant results.

sion, size of tumor, and skin involvement) and lymph node metastasis.

Nottingham prognostic index

Nonparametric χ2 test was used to analyze the association of CK2α with Nottingham prognostic index of patients. Total CK2 scores were significantly associated with the increasing prognostic index (p<0.0001), Table II. Data was split into cytoplasmic and nuclear staining scores. Cytoplasmic component did not show any significant association with Nottingham prognostic index of patients. But nuclear staining showed a significant (χ2 test p<0.0001) association with the increasing Nottingham prognostic index of patients. Data was stratified in to two groups. Group one was with Nottingham prognostic index 3-5 and the other was 6-9. Further samples were assigned to each group on the basis of nuclear staining present (with score of Nu0/1+) or nuclear staining greater than +1 (i.e. Nu+2/+3). Similar groups were assigned to cytoplasmic staining scores. For total CK2α staining the two groups were; group one with score of +1 and +2 and second group included cases with score of 3+ and above, Table II

Lymph node metastasis

To assess the role of CK2α with the metastasis of breast cancer, χ2 test was carried out. Statistics showed a strong positive association of total CK2α scores with lymph node metastasis (χ2 p=0.0001). When data was split into cytoplasmic and nuclear stain scores only nuclear staining score was found to be significantly related to metastasis of the disease (χ2 p<0.0001), Table III.

Size of Tumor (cm³), Lymphovascular invasion and skin involvement

No significant association or correlation could be drawn between total and cytoplasmic CK2α scores and lymphovascular invasion. Whereas nuclear staining was found to be significantly associated with lymphovascular invasion (p=0.002). Table IV

Regression analysis was carried out to determine the relationship between CK2α and size of the tumor in cm³. None of the variable showed any significant association with the size of the tumor, Table IV.

No significant association could be drawn between CK2α scores and skin involvement. Even after stratification of data into nuclear and cytoplasmic staining. No significant inference could be drawn between these parameters. Table IV

DISCUSSION

Present study reports that over all expression of CK2α is significantly associated with the increasing Nottingham prognostic index in the primary breast cancer tissue specimens. We have observed that nuclear localization of the CK2α is critical in this regard and is strongly co-related with increasing prognostic index. Previous literature supports our findings. Diffused CK2 localization has been reported in normal cells but in cancer cells, CK2 is intensely localized in nuclear compartment.[16] Previously there were independent efforts relating the CK2α with poor prognosis[1,10-14] but to our knowledge there is very little or almost no data available which correlates CK2α with NPI. Many other immunohistochemical markers have been associated with the Nottingham prognostic index.[17] Various studies in many other forms of cancer also reports CK2α as an important prognostic marker. The global profiling of gene expression shows that CK2 has been marked as a marker for prognosis in the patients having lung squamous cell carcinoma.[18] CK2 activity elevation has been observed and related to poor out comes in human prostate and breast cancer.[1,11] In head and neck cancer, elevated CK2α has also been associated with poor prognosis and aggressive form of the disease.[19] Thus our study is in line with the previous findings that supports the role of CK2α as a potential prognostic marker and a potent therapeutic target against cancer[19-22] and adds to the existing knowledge about the role of CK2α in prognosis of cancer. CK2α gene has now also been added to the list of 'invasiveness gene signature'. This list comprise of 186 genes, associated with metastasis and poor survival rate in breast cancer.[23]

We propose that distribution pattern of CK2α is critical in progression of

the disease and CK2α is a promising potential prognostic marker in breast cancer. The present study has also shown that high levels of CK2α are associated with lymph node metastasis and lymphovascular invasion. Previous studies have also linked CK2α over expression with various parameters predicting aggressiveness of the tumor.[1,4-5,10,12,18] Development of metastasis is linked with CK2α over expression in breast cancer.[1] Over expression of CK2α has been significantly linked with T3-T4 stage and local invasion in colorectal cancer.[24] In acute myeloid leukaemia over expression of CK2 has been associated with poor clinical outcome.[25] Thus our findings are in line with the previous studies and our data also supports the role of CK2α in development of the aggressive form of tumor. Taken together; with these two findings we can say that CK2α can serve as a potent marker in identification of a sub set of the patients that are at higher risk of developing distant metastasis and may need a more specific and aggressive management and treatment.

CONCLUSION

Keeping in view the above findings and the fact that CK2α has more than 300 substrate[26] and is responsible in manipulating many signaling pathways involved in pathogenesis of breast cancer[1,6] our data supports the notion in favor of CK2α as prognostic marker in breast cancer. Present study considerably adds to the existing knowledge about the role of CK2α in prognosis of breast cancer and we have shown that CK2α has a clear advantage over routine histopathological parameters in predicating the behavior of the cancer cells. Especially the nuclear component of the CK2α holds the key in this respect. As our data has shown that even if the total or cytoplasmic levels of CK2α might behave similarly but nuclear staining is clearly deregulated and is associated with the more aggressive form of the tumor. Thus, CK2α might be helpful in further classification of the patients into sub-groups. We support the inclusion of CK2α into prognostic set of markers used currently in clinical practice. This inclusion will be helpful to identify reliably sub set of breast cancer patients that are at higher risk of developing distant metastasis and needs specific and personalized treatment.

Although present study is a step ahead in the understanding of the basic CK2 biology and its potential role in the prognosis of the breast cancer; further large scale and multicenter studies are required to firmly establish its role. Such expended trials will evaluate CK2 status in many other important aspect of the cancer such as recurrence and survival rate after treatment. Large studies will also take into account the demographic differences (if any) in expression of CKα in various population groups. After robust epidemiological studies and clinical trials CK2α will be firmly established as a prognostic marker.

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AUTHOR'S CONTRIBUTION

Following authors have made substantial contributions to the manuscript as under:

FQ: Concept, acquisition & interpretation of data, drafting the manuscript, final approval of the version to be published

AKN: Study design, Supervision, critical revision, final approval of the version to be published

SS: Acquisition, analysis & interpretation of data, final approval of the version to be published

PW: Drafting the manuscript, final approval of the version to be published

IM, RY, SNH: Acquisition of data, final approval of the version to be published

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

CONFLICT OF INTEREST

Authors declare no conflict of interest

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KMUJ web address: www.kmuj.kmu.edu.pk

Email address: kmuj@kmu.edu.pk