Original paper

Nuclear expression of Ku70/80 is associated with *CHEK2* germline mutations in breast cancer

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Ku70/80 protein inhibitors reduce the repair of DNA double-strand breaks via the Ku70/80 pathway, so they can be used to treat cancers with Ku70/80 overexpression. Since the association of Ku70/80 with germline *CHEK2* mutations in breast cancer is unknown, in this study we evaluated the expression of Ku70/80 in breast cancers with germline *CHEK2* mutations.

Immunohistochemistry with a Ku70/80 antibody on tissue microarrays from 225 *CHEK2*-associated breast cancers was used and automatically assessed with computerized image analysis.

We report that the vast majority of breast cancers expressed high level of nuclear Ku70/80 and a small percentage of tumors (3.5%) were negative for Ku70/80 expression. There was a significant difference between the nuclear Ku70/80 expression in *CHEK2*-associated vs. *CHEK2*-non-associated breast cancers in all tumors ($p = 0.009$), and in the estrogen receptor (ER) positive subgroup of breast cancers ($p = 0.03$).

This study is the first reporting an association of Ku70/80 expression with *CHEK2* germline mutations in breast cancer. The results suggest that evaluation of Ku70/80 expression in breast cancer may improve the selection of breast cancer patients for Ku70/80 inhibitor therapy, and point to *CHEK2*-associated breast cancer and a subset of ER-positive breast cancer as potential suitable targets for such therapy.

Key words: breast cancer, *CHEK2*, Ku70/80.

Introduction

Recent advances in understanding of the mechanisms involved in DNA damage signaling and repair have opened up a new avenue in treatment of breast cancer, i.e., DNA repair targeted therapy, which kills cancer cells preventing DNA repair [1–3]. A spectacular example of this approach to treatment of breast cancer is application of a poly (ADP-ribose) polymerase (PARP) inhibitor, such as olaparib, to patients with germline *BRCA1* mutations [4, 5].

Ku70/80 is a heterodimer of two polypeptides, Ku70 (encoded by *XRCC6*) and Ku80 (encoded by *XRCC5*), that binds to DNA double-strand break (DSB) ends and is necessary for the non-homologous end joining (NHEJ) pathway of DNA repair [6]. Ku70/80 forms a complex with the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to form the full DNA-dependent protein kinase, DNA-PK [7]. Experiments with Ku70/80 knocked out mice indicate that NHEJ is important for genome maintenance [8]. In addition to its role in NHEJ, Ku70/80

is also required for telomere length maintenance [9] and other important cellular mechanisms. Deletion or mutation of genes coding for Ku70 or Ku80 proteins results in a highly radiosensitive phenotype [10].

It has been reported that Ku-DNA binding inhibitors modulate the DNA damage response in response to DNA DSB [11]. Ku-DNA binding inhibitors that specifically block the Ku70/80 heterodimer interaction with DNA have been developed [12–14]. These inhibitors inhibit NHEJ-catalyzed DSB repair and sensitize *in vitro* cancer cells to DSB-inducing agents. Therefore, Ku-DNA binding inhibitors seem to be a promising anticancer therapeutic strategy in combination with DNA DSB-inducing agents [11].

Checkpoint kinase 2 (*CHEK2*), a cell cycle checkpoint regulator gene, codes for a kinase protein activated in response to radiation and other agents that cause breaks in the DNA. *CHEK2* is not only a breast cancer susceptibility gene but also a multi-organ cancer susceptibility gene [15]. It is a tumor suppressor gene, involved in DNA DSB repair through the process of homologous recombination [16]. It is also involved in cell cycle arrest and apoptosis [17].

There are only a few reports on the expression of Ku70/80 in breast cancers [17–21], but the association of this protein with germline *CHEK2* mutations in breast cancer patients is unknown. Germline *CHEK2* mutations have been associated with estrogen receptor (ER) positive breast cancer [22, 23]. However, although ER positive tumors tend to have a better prognosis in unselected breast cancer patients, germline *CHEK2* mutations are associated with increased risk of the development of ER positive breast cancer with an unfavorable prognosis [24–26].

In order to identify breast cancer patients who could potentially benefit from Ku-DNA binding inhibitors, we looked for a subgroup of breast cancer that would be associated with Ku70/80 expression. Defining such a subgroup could help accurately select breast cancer patients for clinical trials using Ku-DNA binding inhibitors. To this end, we decided to test breast cancers for expression of Ku70/80 protein and germline mutations in *CHEK2*.

Table I. Correlation of Ku70/80 expression with clinicopathological parameters and germline mutations in *CHEK2* and *BRCA1*

Parameters	*n*	Ku70/80 (median)	*p*-value
Age			0.9
< 50	64	211.3	
≥ 50	161	202.4	
Grade			0.78
1	21	203.2	
2	128	197.2	
3	76	215.1	
Tumor size [cm]			0.59
≤ 2	138	209.3	
> 2	87	199	
Nodal status			0.36
Negative	145	202.4	
Positive	74	211.6	
ER			0.04
Negative	52	179.6	
Positive	173	208.9	
PR			0.48
Negative	78	197.2	
Positive	147	206.7	
HER2			0.45
Negative	173	203.1	
Positive	49	219	
Triple-negative			0.76
Negative	201	205.7	
Positive	24	212.7	
CHEK2 mutation			0.009
Negative	189	198.7	
Positive	36	224.1	
BRCA1 mutation			0.74
Negative	218	214.1	
Positive	7	204.9	

ER – estrogen receptor, PR – progesterone receptor

Materials and methods

Patients

This study included 225 unselected patients with primary breast cancer (median age: 55 years; range: 27–91 years) diagnosed between 2010 and 2014 at the International Hereditary Cancer Center in Szczecin. Patients did not receive endocrine therapy, chemotherapy, or radiotherapy before surgery. None of the cases were selected based on family history. The clinicopathological patient characteristics are shown in Table 1. The study was approved by the Ethics Committee of the Pomeranian Medical University in Szczecin, Poland.

Genotyping

Genomic DNA was prepared 5–10 ml of peripheral blood. Mutation analysis for the common germline mutations in the Polish population was performed as described previously [15, 27, 28]. The *CHEK2* del5395 mutation was detected by a multiplex polymerase chain reaction (PCR) reaction. The c.444+1G > A and c.470T > C vari-

ants in *CHEK2* were detected using restriction fragment length polymorphism PCR analysis, and the c.1100delC mutation was analyzed using allele specific oligonucleotide PCR. Furthermore, patients were tested for three founder germline mutations in *BRCA1* (C61G, 4153delA, 5382insC) described in the Polish population [29, 30]. For samples in which a mutation was detected, a separate DNA sample was sequenced to confirm the presence of the mutation. To avoid false results in all reactions, positive and negative controls (without DNA) were used.

Tissue microarray construction

We collected all available paraffin blocks containing sufficient tumor tissue from primary breast cancers. From each case, a representative tissue block consisting of predominantly tumor tissue was selected for tissue microarray construction as previously described [31]. Three different regions in the outer invasive margin of the tumors were identified and marked on hematoxylin and eosin stained sections. Sections were matched to their corresponding wax blocks (the donor blocks), and three 0.6-mm diameter cores of the tumor tissue were removed from the donor blocks and inserted into the recipient master block using a tissue microarrayer (Beecher Instruments, Silver Spring, MD). The recipient block was cut and sections were transferred to coated slides. One slide was stained with hematoxylin and eosin and a subsequent slide for immunohistochemistry.

Immunohistochemistry

The slides were immunostained using a Dako EnVision FLEX+ visualization system with an automated immunostainer (Dako Autostainer Link 48) according to the manufacturer's instructions. Mouse monoclonal Ku70/80 antibody (Abcam, Cambridge, United Kingdom) was used (clone 162; dilution 1 : 100; incubation time 30 min). The reaction was developed with a diaminobenzidine substrate-chromogen solution and counterstained with hematoxylin. Appropriate positive and negative controls were run.

Image analysis

Image analysis was performed similarly as described previously [32]. Microscope slides were scanned using an Aperio ScanScope CS (Aperio, Vista, CA, USA) using a 20×/0.75 Plan Apo lens. The scanned images were analyzed with ImageScope (Aperio) software using an algorithm for automatic analysis of the immunochemistry marker's expression (Aperio IHC nuclear image analysis algorithm v. 9.0). Tumor areas were marked manually for analysis (Fig. 1C, D). Furthermore, tumor cells were automatically discriminated from benign cells (e.g., lymphocytes, fibroblasts) based on size and shape (roundness, compactness, elongation) algorithm parameters. The intensity of the nuclear staining and the percentage of Ku70/80-positive cells were determined automatically. The image analysis algorithm parameter "nuclear stain only" was selected to generate markup images. Brown (DAB) and blue (hematoxylin) nuclei were identified and were spectrally separated with ImageScope software to evaluate immunohistochemical markers.

Immunohistochemistry scoring

The results were reported using an H-score [33] that takes into account both staining intensity and the percentage of positive cells according to the following formula: H-score = (% of cells stained at intensity category 1 × 1) + (% of cells stained at intensity category 2 × 2) + (% of cells stained at intensity category 3 × 3). We subdivided the H-scores into categories: low scores < 100, medium scores 100–200, and high scores 201–300. Tumors were considered HER2 positive if scored as 3+ or 2+ with amplification tested by FISH [34]. Estrogen receptor and progesterone receptor (PR) were considered positive if staining was detected in ≥ 1% of nuclei [35].

Statistical analysis

Analysis of differences in distributions of Ku70/80 expression between two groups of patients was performed using the Mann-Whitney *U* test and differences between more than two groups were evaluated by the Kruskal-Wallis test. Statistical analyses were performed using GraphPad Prism v. 9.1 (GraphPad Software, La Jolla, CA, USA). $P < 0.05$ was considered significant.

Results

Immunohistochemical staining with the Ku70/80 antibody revealed a strong nuclear reaction in tumor cells (Fig. 1B, D) in the majority of breast carcinomas; however, there were also Ku70/80 negative cases (Fig. 1A, C). The distribution of Ku70/80 H-scores among 225 breast cancers is shown in Figure 2. The H-scores ranged from 0 to 289. The median and mean H-scores were 206 and 179, respectively. High (H-score > 200), medium (H-score 100-200), low (H-score < 100), and negative (H-score < 1) expression of Ku70/80 was found in 52.4% (118/225), 28.9% (65/225), 18.7% (42/225), and 3.5% (8/225) of breast cancers, respectively. Ku70/80 expression could also be found in the nuclei of lobules, some lymphocytes and stromal cells.

There was a statistically significant association between Ku70/80 and status of ER and *CHEK2* germline mutation. Higher expression of Ku70/80 was associated with positive ER (median 179.6 vs. 208.9, $p = 0.04$) and the presence of germline mutations

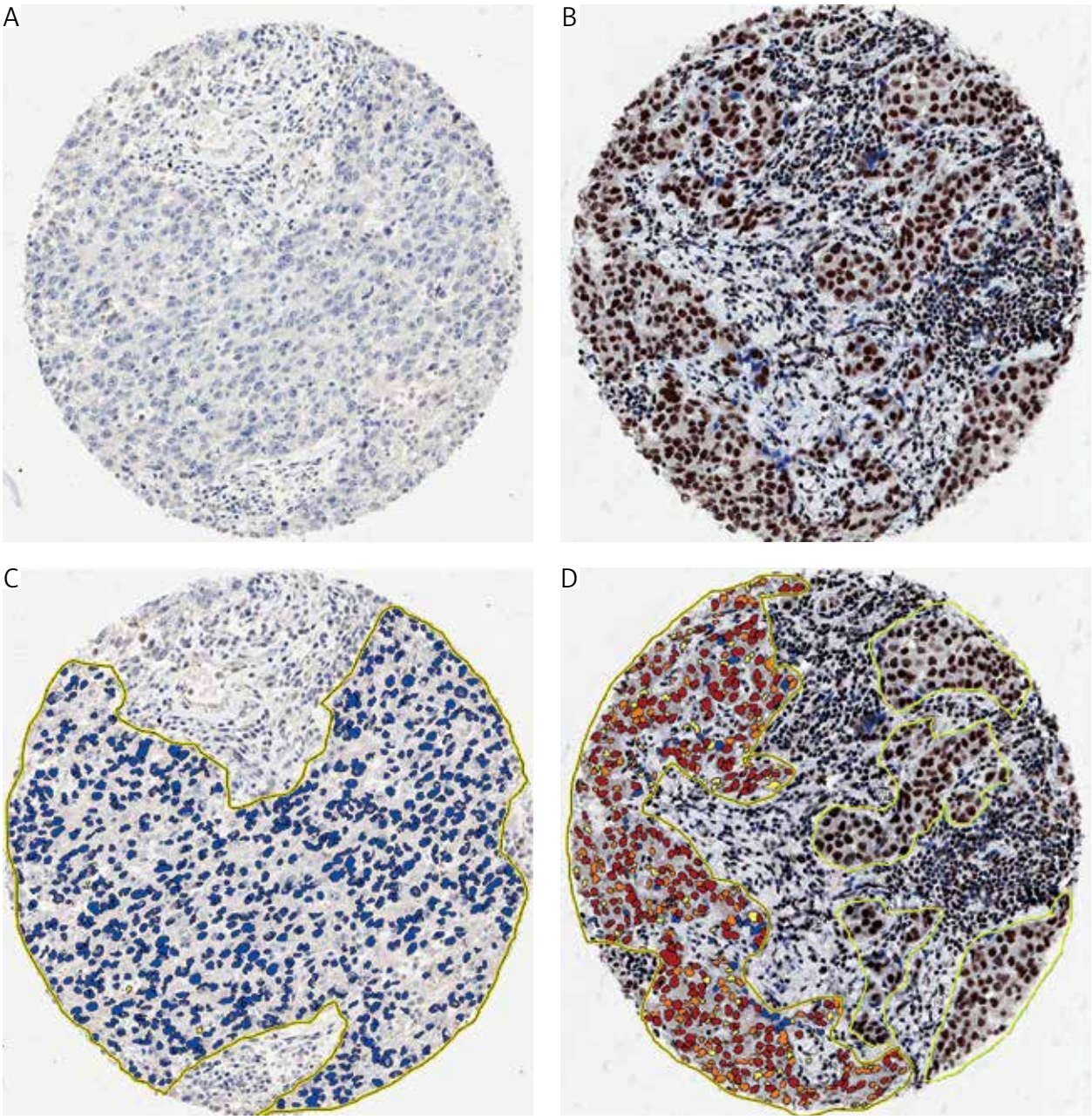

Fig. 1. Image analysis of Ku70/80 expression in representative examples of Ku70/80 positive (**B, D**) and negative (**A, C**) breast cancers. Blue nuclei were counted as negative (0) and brown nuclei (DAB+) as positive. The intensity of the brown nuclear staining was automatically scored as low, moderate, and high (yellow, orange, dark red colors respectively in D)

in *CHEK2* (median 198.7 vs. 224.1, $p = 0.009$). Similarly, among the subgroup of ER-positive breast cancers, high Ku70/80 expression was associated with the presence of germline mutations in *CHEK2* ($p = 0.03$). No other statistically significant associations between Ku70/80 expression and clinicopathologic characteristics were found (Table 1).

Discussion

The successful treatment of breast cancer requires the precise selection of a target group of patients who are most likely to benefit from the applied therapy. In this report we document that nuclear expression of Ku70/80 is associated with *CHEK2* germline mutations in breast cancer. We found high and medium levels of expression of Ku70/80 in 81% of breast cancers with *CHEK2* mutations. A similar relationship was present in the subgroup of cancers with ER expression, in which high Ku70/80 expression was also associated with the presence of *CHEK2* germline mutations. These results suggest that germline *CHEK2* mutations select a group of breast cancers that may benefit from Ku70/80 inhibitor therapy.

Recently, inhibitors of the Ku70/80 protein and the NHEJ pathway have been reported [12, 14].

They can reduce DSB repair via Ku70/80 and can be used for the treatment of cancer with overexpressed or upregulated Ku70/80. Therefore, our results suggest that breast cancer patients with germline *CHEK2* mutations may be potential candidates for treatment with Ku70/80 inhibitors (in a synthetic lethality mechanism). Ku70/80-DNA binding is the first step of the NHEJ pathway so the Ku70/80 heterodimer protein is an indispensable element in the NHEJ mechanism and a logical target for disruption of the entire NHEJ pathway. The non-homologous end joining pathway is frequently upregulated in tumor cells in order to compensate for DSB repair defects or for innate genomic instability; therefore components of this pathway can constitute a target for synthetic lethality based treatment, especially in combination with DSB inducing ionizing radiation or chemotherapeutic agents [36].

It is likely that the high level of expression of Ku70/80 in breast cancers with *CHEK2* mutations is due to the increased demand for DNA repair by the NHEJ mechanism, because in these cancers DNA repair by the homologous recombination pathway is impaired. Inhibitors of Ku70/80 could reduce expression of this heterodimer protein and make cancer cells with *CHEK2* germline mutations susceptible to DSB inducing therapy. However, our results also revealed a small subset of *CHEK2*-associated cancers (3.5%) with negative Ku70/80 expression. Patients with these cancers may either not benefit from this type of therapy or may benefit very little because of a lack or low level of the therapeutic target.

Ku70/80 has emerged as a possible therapeutic target in the treatment of a variety of cancers with the use of chemotherapy or radiotherapy. Increased expression of the Ku subunits is associated with radioresistance of nasopharyngeal [37] and oral cavity [38] cancers. Upregulation of the NHEJ proteins Ku70/80 has been reported in radioresistant cervical cancer [39]. XRCC5 (Ku80) was one of 50 genes showing overexpression in the radioresistant cervical squamous carcinomas relative to the radiosensitive ones [40]. The higher expression of this gene in radioresistant cancer cells is consistent with its physiological function, as XRCC5-deficient cells and Ku80-knockout mice are hypersensitive to ionizing radiation [41, 42].

Conversely, downregulation of Ku70/80 genes and reduced expression of Ku70/80 proteins have been associated with a better response to radiotherapy or chemotherapy. Cervical cancer patients with low expression of Ku80 respond better to radiotherapy [43]. Hypopharyngeal squamous cell carcinoma with low Ku70 or XRCC4 proteins has better sensitivity to chemoradiotherapy [44]. Previously, we reported that XRCC5 (Ku80) and XRCC6 (Ku70) were 2 of 25 genes showing downregulation which was significantly associated with a pathological complete response after cisplatin neoadjuvant therapy in *BRCA1*-associated triple-negative breast cancers [45].

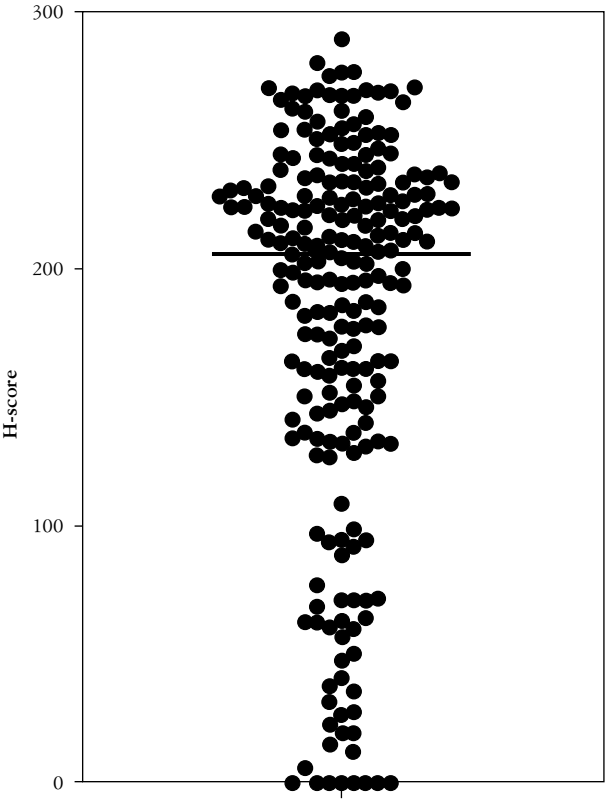

Fig. 2. Scatter dot plot of the distribution of H-scores among 225 breast cancers. The solid line represents the median

There are only a few reports on the expression of Ku70/80 in breast cancers [17–21]. In our study, in the entire study group, high and medium expression of nuclear Ku70/80 (marker of NHEJ) was found in 52.4% and 28.9% of breast cancers respectively (81.3% altogether). Others reported 40–87% of breast cancers with high expression of Ku70/80 [17, 20, 21]. In our study there was no association of Ku70/80 expression with clinico-pathological factors such as tumor grade, pT, pN, and patient age. Similar results were obtained by others [21]. However, whereas Agboola *et al*. [21] reported an inverse correlation between expression of Ku70/80 and expression of ER, PR, HER2, and BRCA1, and a positive correlation of Ku70/80 expression with triple negative breast cancers, we did not find correlations with expression of PR, HER2, and with *BRCA1*-associated and triple negative breast cancers. On the other hand, we found a positive correlation of expression of Ku70/80 with the presence of *CHEK2* germinal mutations and with positive ER. We also noted that high levels of Ku70/80 expression were associated with ER+ *CHEK2*+ phenotype as opposed to ER+ *CHEK2*- phenotype.

The differences can be attributed to different antibodies used, different areas of tumor tissue taken

for evaluation and different methods of assessment of Ku70/80 positive cells. In contrast to the subjective manual evaluation methods used in previously published studies [18–21], in our work we used an objective method, i.e. automatic computerized image analysis, to assess the expression of Ku70/80 in the nuclei of cells. We tested three different regions in the outer invasive margin of tumors. In other reports, tumor cores were taken from the peripheral and central portions of tumor tissue [20, 21].

Conclusions

This study is the first reporting an association of Ku70/80 expression with *CHEK2* germline mutations in breast cancer. Our findings have two major implications. First, they point to breast cancers with mutations in *CHEK2* as a subgroup that may be a suitable target for treatment with Ku70/80 inhibitors, and should therefore be considered when organizing clinical trials of these inhibitors. Second, the results showed that high levels of Ku70/80 expression were associated with ER+ *CHEK2*+ phenotype as opposed to ER+ *CHEK2*- phenotype, suggesting that the therapeutic results of Ku70/80 inhibitor use may be different in these subgroups of estrogen positive breast cancer. As such, these findings are important for planning future clinical trials with Ku70/80 inhibitors and analyzing their results, as they isolate subgroups of ER positive breast cancer that may respond differently to such therapy.

Acknowledgments

This study was supported by the Pomeranian Medical University Research Program, grant no. SPN-125-01/19.

The authors declare no conflict of interest.

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